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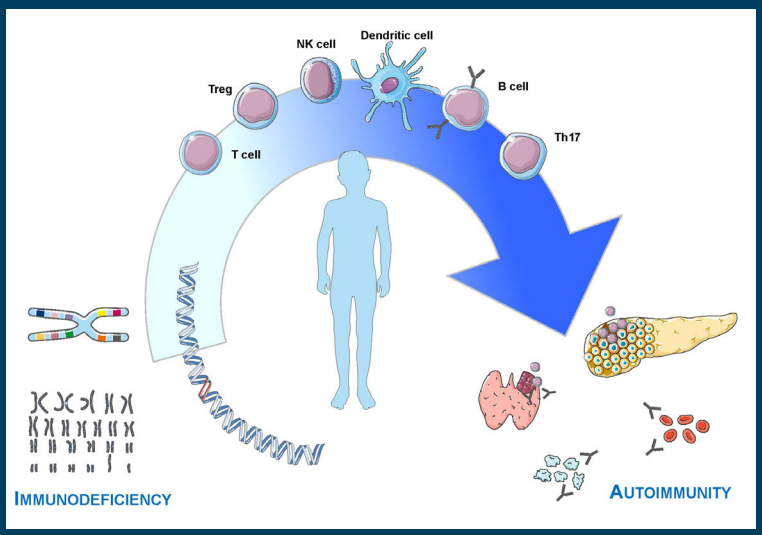
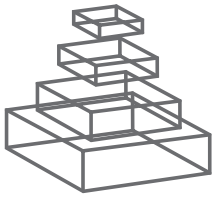


AUTOIMMUNITY AND IMMUNODEFICIENCY

Topic Editors
Luigi D. Notarangelo and Rosa Bacchetta

frontiers in IMMUNOLOGY



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ISSN 1664-8714
ISBN 978-2-88919-164-2
DOI 10.3389/978-2-88919-164-2

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AUTOIMMUNITY AND IMMUNODEFICIENCY

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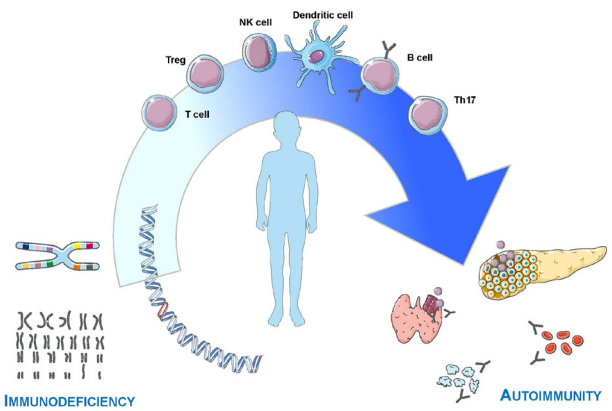

Image created by Aisha Sauer and Luigi D. Notarangelo using Servier Medical Art.

Immune regulation results from a finely tuned network of distinct mechanisms operating throughout life and balancing the need to clear infections and prevent self-aggression. Primary Immunodeficiencies (PIDs) are "experiments of nature" where the ability to fight against pathogens is deeply impaired. The study of patients with PIDs has been instrumental to identify and characterize key components and mechanisms that govern development and function of the human immune system. Recently, it has become clear that in congenital monogenic diseases the ability of the immune system to build and maintain active tolerance to self can be specifically altered, so that autoimmune symptoms may easily prevail over infections in these pathologies. In addition, increasing observations have brought the attention to the fact that hypomorphic mutations in genes that control T and/or B cell development are often associated with clinical and laboratory features of immune dysregulation, thus expanding the spectrum of PID phenotypes. For example, mutations in genes driving T cell development can lead to defective lymphostromal cross-talk in the thymus and impinge of negative selection of self-reactive T cells and/or Treg function. Similarly, disorders of B cell development may associate with defects of receptor editing and/or with abnormalities of peripheral B cell homeostasis. On the other hand, autoantibodies can provoke defective immune responses by targeting cytokines and/or immune cells.

This Research Topic will focus on i) summarizing updated clinical and immunological features of diseases characterized by immune dysregulation of known and still undefined origin and ii) gathering new insights into the mechanisms of T and B cell development, function and interaction, in order to broader the comprehension of the pathogenesis of autoimmunity and to ultimately advance the definition of novel therapeutic strategies.

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IMMUNOLOGY

EDITORIAL
published: 12 April 2013
doi: 10.3389/fimmu.2013.00077

Immunodeficiency with autoimmunity: beyond the paradox

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The association of immunodeficiency and autoimmunity may represent a paradox, yet it has been described in an increasing number of conditions. Use of unbiased genomic approach to identify novel forms of primary immunodeficiencies (PIDs), along with in-depth functional studies in biological samples from affected individuals continue to unravel novel mechanisms underlying immune dysregulation in patients with altered ability of fighting pathogens. In particular, it has been clearly established that genetic defects that affect T and B cell development compromise not just the ability to generate a diversified repertoire of lymphocytes capable of recognizing multiple pathogens, but also impinge on mechanisms of central and peripheral tolerance, hence favoring autoimmune and inflammatory manifestations.

Yet, the diagnosis of autoimmune symptoms in the context of PIDs is troublesome, the prognosis unclear, and the treatment challenging. In the present collection of manuscripts, several experts in the field provide an overview of the spectrum of different forms of monogenic defects of the immune system manifesting also with autoimmunity, and discuss established and novel mechanisms involved in immune dysregulation.

Studies on patients with Immunedysregulation-Polyendocrinopathy-Enteropathy-X-linked (IPEX) Syndrome, have paved the way to understand the phenotype arising from impaired peripheral tolerance due to dysfunctional regulatory T cells (Treg) expressing mutated FOXP3. However, this important T cell subpopulation can also be affected in other forms of PID, such as Wiskott–Aldrich syndrome (WAS) and adenosine deaminase (ADA) deficiency. In these disorders, the underlying genetic defect affects multiple cell types, resulting in impaired immune defense, but also poor Treg function. Similarly, *STAT5B* mutations disrupt an essential intracellular transcriptional activator for Treg cells, causing reduction of Treg number in affected individuals.

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is an autosomal recessive condition due to mutation of the Autoimmune regulator (*AIRE*) gene. Patients with APECED present with predominant organ specific autoimmunity and autoantibodies with multiple specificities. AIRE has been shown to play a critical role in allowing expression of self-antigens in the thymus, thereby permitting deletion of self-reactive T lymphocytes or their diversion to Treg cells. Thus APECED stands as the prototypic monogenic disorder of central T cell tolerance. While it is still questionable whether deficiency of AIRE also affects peripheral tolerance, recent data indicate that the autoimmune-associated tissue damage may not be primarily due to autoantibodies, but rather to autoreactive $CD8^+$ T cells.

Moreover, recent studies in patients affected with Common Variable Immunodeficiency, a condition in which proper specific antibody production is deficient in favor of pathogenic autoantibody secretion, have highlighted the importance of mechanisms that control B cell development and receptor editing in maintaining immune homeostasis.

Finally, two manuscripts call the attention to the dual role of certain cell types and their ability to acquire different immunological functions depending on the environment in which they differentiate, as described for Th17 cells and dendritic cells, at the end of the Topic. Possibly, the future of medicine should aim to implement physiological plasticity and to empower epigenetics modifications in order to recover from inborn errors of Nature.

Received: 19 February 2013; accepted: 09 March 2013; published online: 12 April 2013.
*Citation: Bacchetta R and Notarangelo LD (2013) Immunodeficiency with autoimmunity: beyond the paradox. Front. Immunol. **4**:77. doi: 10.3389/fimmu.2013.00077*
This article was submitted to Frontiers in Primary Immunodeficiencies, a specialty of Frontiers in Immunology.

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IMMUNOLOGY

REVIEW ARTICLE
published: 31 July 2012
doi: 10.3389/fimmu.2012.00211

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: a paradigm of immunodeficiency with autoimmunity

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Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a rare monogenic primary immunodeficiency (PID) due to mutations of *FOXP3*, a key transcription factor for naturally occurring (n) regulatory T (Treg) cells. The dysfunction of Treg cells is the main pathogenic event leading to the multi-organ autoimmunity that characterizes IPEX syndrome, a paradigm of genetically determined PID with autoimmunity. IPEX has a severe early onset and can become rapidly fatal within the first year of life regardless of the type and site of the mutation. The initial presenting symptoms are severe enteritis and/or type-1 diabetes mellitus, alone or in combination with eczema and elevated serum IgE. Other autoimmune symptoms, such as hypothyroidism, cytopenia, hepatitis, nephropathy, arthritis, and alopecia can develop in patients who survive the initial acute phase. The current therapeutic options for IPEX patients are limited. Supportive and replacement therapies combined with pharmacological immunosuppression are required to control symptoms at onset. However, these procedures can allow only a reduction of the clinical manifestations without a permanent control of the disease. The only known effective cure for IPEX syndrome is hematopoietic stem cell transplantation, but it is always limited by the availability of a suitable donor and the lack of specific guidelines for bone marrow transplant in the context of this disease. This review aims to summarize the clinical histories and genomic mutations of the IPEX patients described in the literature to date. We will focus on the clinical and immunological features that allow differential diagnosis of IPEX syndrome and distinguish it from other PID with autoimmunity. The efficacy of the current therapies will be reviewed, and possible innovative approaches, based on the latest highlights of the pathogenesis to treat this severe primary autoimmune disease of childhood, will be discussed.

Keywords: IPEX, FOXP3, Treg, autoimmune enteropathy, neonatal diabetes, neonatal eczema, HSCT

INTRODUCTION

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a rare monogenic primary immunodeficiency (PID), characterized by multi-organ autoimmunity. It is caused by mutations in the transcription factor *forkhead box p3* (*FOXP3*), the master gene of T regulatory (Treg) cells. The disease shows an X-linked hereditary pattern: only males are affected, whereas the carrier mothers are healthy.

Although IPEX syndrome is a rare disease, the recent increase in the number of patients referred for diagnosis suggests that the occurrence of the disease has been underestimated so far. At present, 63 *FOXP3* mutations have been published, for an overall number of 136 patients described, and of these about half have been diagnosed in the last 3 years. This also indicates that the awareness of the disease has been growing with a better understanding of the role of FOXP3 and Treg cells in maintaining peripheral tolerance.

Overall, the analysis of cases reported so far (**Table 1**) confirms the relevance of the three main clinical manifestations and their early onset while highlighting the occurrence of unusual symptoms. The genetic analysis is always required for accurate diagnosis, although other tests such as tissue biopsy and/or autoantibody detection are important, as complementary tools, in the diagnostic process and follow-up.

IPEX syndrome can be fatal in early infancy if not recognized, therefore a timely diagnosis is essential to start appropriate treatment. Treating IPEX patients poses a threefold challenge: autoimmunity, infections supported by the autoimmune damage, and the severity of the overall picture. Both novel and existing therapeutic approaches will be discussed with an emphasis on the central role of Treg cell impairment in the pathogenesis of IPEX syndrome.

GENETICS OF IPEX SYNDROME

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome was described for the first time in 1982 in a large family with 19 affected males across five generations, as an X-linked syndrome with diarrhea that was lethal in most male infants by the first months or years of life (Powell et al., 1982). Only 20 years

Table 1 | Clinical features, therapy and outcome in reported IPEX patients.

Reported by	Pt°	Mutation	Age at onset	Age at diagnosis	Diarrhea	T1DM	Eczema	Eos (/mm^3); IgE (IU/mL)	Additional clinical findings	Therapy	Outcome♦
Peake et al. (1996), Wildin et al. (2001)	V6^f, 2^f	c.1290_1309 del_insTGG	6 weeks	Post mortem	+	+	+ Peeling skin	3170; 9999–40,000 μg/L	Anemia, lymphadenopathy, sepsis	TPN, plasma IV	Exitus 10 months
Ferguson et al. (2000), Bennett et al. (2001b), McGinness et al. (2006)	F1-V1^f, V2^f, 1^f	c.1150G>A	1 month	9 years	+	−	+	230–900; 755–3492	Pemphigoid nodularis; bullous pemphigoid; infections, asthma	PD, CSA, dapsone, IgIV, rituximab	Alive 14 years
Ferguson et al. (2000), Bennett et al. (2001b)	F1-IV10^f	c.1150G>A$^\#$	2 months	−	− (Vomiting)	−	+	936; 250	Hypothyroidism; infection	na	Exitus 10 months
	F1-V4^f	c.1150G>A$^\#$	2 weeks	−	+	+	+	620; 30	Hypogammaglobulinemia, infections, sepsis	IgIV	Exitus 2 years
Ferguson et al. (2000), Bennett et al. (2001b)	F1-V5^f, V7^f	c.1150G>A	3 weeks	Post mortem	−	+	+	500; 22	Sepsis	IgIV, CSA	Exitus 12 weeks
Chatila et al. (2000)	3-F1; F2^f −14, 15, 27, 28	c.1044 +4A>G; c.750_752 delGGA	3 weeks −3 months	na	5/5	5/5	4/5	na; 4/5 hyper IgE	Autoimmune cytopenia 3/5, food allergy5/5	na	Exitus 1/5, alive 4/5
Levy-Lahad and Wildin (2001), Wildin et al. (2001)	1^f, F1^f	c.1189C>T$^\#$	Birth	−	− (Atonic gut)	−	+	na	Hypotonia, hypothyroidism, thrombocytopenia, peritonitis, cholangitis	−	Exitus 19 days
	2^f, F1^f	c.1189C>T	Birth	Post mortem	− (Ileo)	+	−	na	Cachexia, hypotonia, thrombocytopenia, peritonitis	−	Exitus 5 weeks
	3^f, F1^f	c.1189C>T$^\#$	Birth	−	+	+	−	na	Infections, sepsis	Dexamethasone, CSA	
Wildin et al. (2001)	3na	c.1113T>G	na	na	+	+	+	na	Anemia	HSCT	Alive, age na

	4[na]	c.1150G>A	na	na	+	+	+	na	Hypothyroidism, thrombocytopenia, sepsis	na	Exitus 4 months
Bennett et al. (2001b), Kobayashi et al. (2011)	F2, 5	c.1293_1294 delCT	na	na	+	−	−	na	−	HSCT	Exitus age na
Kobayashi et al. (2001, 2011), Fuchizawa et al. (2007), Otsubo et al. (2011)	1[f], 1[f], 3, 1[f]	c.227delT	15 days	na	+	−	−	na; +	Thyroiditis, AHA, tubulonephropathy*	FK506, betamethasone	Alive 19 years
Kobayashi et al. (2001, 2011)	2[f], 4[f]	c.1087A>G	na	na	+	+	−	na; +	Thyroiditis, tubulonephropathy*, infections, sepsis	na	Exitus 3 years
Baud et al. (2001)	1[f]	c.1113T>G[#]	na	−	+	+	Ichthyosis	na	ITP	na	Exitus 4.5 months
	2[f]	c.1113T>G	1 month	4 months	+	+	Ichthyosis	na; 1750	ITP, AHA, autoimmune neutropenia, cholestatic hepatitis	MPD, FK506, HSCT	Exitus 2 years 7 months
Wildin et al. (2002), McMurchy et al. (2010)	1[f]	c.1040G>A	3 months	13 years	+	+	−	na	Infection (sepsis)	PD, CSA, FK506, HSCT	Exitus 14 years
Wildin et al. (2002)	2	c.1044 + 459A > G	<1 month	na	+	+	+	na	Lymphadenopathy, hepatosplenomegaly eczema, hypothyroidism, AHA, immune neutropenia, infections	Steroids, rituximab, IgIV	Alive 5 years
	3[f]	c.748_750 delAAG, c.543C>T	2 months	9 years	+	+	−	na	Arthritis, ITP, hepatomegaly, mild hepatitis, progressive renal insufficiency	Steroids, CSA, FK506, rofecoxib, MTX, rituximab, IgIV, HSCT	Exitus 10 years

(Continued)

Table 1 | Continued

Reported by	Pt°	Mutation	Age at onset	Age at diagnosis	Diarrhea	T1DM	Eczema	Eos (/mm^3); IgE (IU/mL)	Additional clinical findings	Therapy	Outcome♦
Owen et al. (2003)	A-1[f]	c.227delT[#]	2 weeks	–	+	–	+	na	Lymphoid infiltration of the pancreas	na	Exitus 6 weeks
	A-2[f]	c.227delT	3 weeks	na	+	–	+	na	Hepatitis	PDN, AZA, CSA	Alive 10 years
Nieves et al. (2004)	1[na]	c.1150G>A	7 months	9 years	+	+	+	+; 33	Alopecia, longitudinal ridging nails, autoimmune neutropenia, severe anemia, subclinical thyroiditis	PD, CSA, IgIV, G-CSF	Alive 11 years
Tanaka et al. (2005), Fuchizawa et al. (2007), Kobayashi et al. (2011), Otsubo et al. (2011)	1, 4, 1, 3	c.1117T>G	2 months	4 months	+	–	–	na; 2895–7275	–	CSA, PD, IgIV, HSCT	Alive 7 years
Bindl et al. (2005)	1[na]	c.968-20A>C	7 years	10 years	+	–	Dermatitis	na; 17,370	CSA induced chronic interstitial nephritis	Steroids, PD, AZA, CSA, MTX, rapa	Alive 15 years
	2[f]	+	<2 months		+	–	+	na; 3000	–	Steroids, FK506, AZA, rapa	Alive age na
	3[f]	+	<2 months		+	–	+	na; 2000	–	Steroids, FK506, AZA, rapa	Alive age na
Mazzolari et al. (2005)	1[na]	promoter region	4 months	<1 year	+	–	+	na; 763	sepsis	MPD, CSA, HSCT	Alive 2 years 4 months
Bacchetta et al. (2006), Gambineri et al. (2008), McMurchy et al. (2010), Passerini et al. (2011b)	1, 12, 12, 12	c.1117-1118TT>GC	neonatal	3 months	+	+	+	768; 8423	–	MMF, HSCT	Alive 9 years

	2 [f], 5[f], 6[f], 5[f], 5[f]	c.543C>T, c.970T>C	neonatal		+	−	+	2780; 374	Allergic asthma	None	Alive 7 years
	3, 2, 1, 2	c.3G>A	Neonatal	<1 years	+	+	+	552; 28,800	Hypothyroidism, lymphadenopathy, hepatosplenomegaly	MPD, CSA, HSCT	Alive 10 years
De Benedetti et al. (2006)	1[f]	c.454 + 4A>G	18 days	22 years	+	−	+	N; 200	Recurrent arthritis, psoriasiform dermatitis, hepatomegaly	PD, MPD, CSA, FK506, infliximab	Alive 22 years
	2	c.323C>T	14 months	7 years	+	−	−	N; 74	Steroid-responsive pneumonia and pericarditis, recurrent arthritis	PDN, PD, AZA	alive 7 years
Myers et al. (2006)	1[na]	c.1-7G>T	1 day	Post mortem	+	[§]	−	na	Hypothyroidism, Infections	na	Exitus 54 days
	2[na]	c.1169G>A	4 days	Post mortem	+	+	+	na	Infections	na	Exitus <2 years
Gavin et al. (2006)	1[na]	c.210_210 + 1GG>AC	na	na	+	+	+	na; high	AHA, ITP	FK506, steroids, TPN	Alive 5 months
	2-p1[f]	c.751_753 delGAG	na	na	+	+	+	na; high	Thyroiditis, AHA	Intermittent steroids	Alive 6 years
	2-p2[f]	c.751_753 delGAG	na	na	+	+	+	na; high	Thyroiditis	FK506	Alive 9 years
	3[f]	g.-6247_-4859del	na	na	+	-	+	na; high	food allergies	FK506	Alive 4 years
Moudgil et al. (2007), Rao et al. (2007)	1, 2	c.303_304 delTT	4 months	6 months	+	+	+	–; 1564	Alopecia, AHA, lymphadenopathy, hypothyroidism, MGN, food allergies, infections	TPN, CSA, PD, rituximab, HSCT	Alive 4 years

(Continued)

Table 1 | Continued

Reported by	Pt°	Mutation	Age at onset	Age at diagnosis	Diarrhea	T1DM	Eczema	Eos (/mm^3); IgE (IU/mL)	Additional clinical findings	Therapy	Outcome♦
Heltzer et al. (2007)	1[na]	c.817-1G>A	birth	Post mortem	+	[§]	Rush	na; 5320	–	FK506	Exitus 79 days
	2[na]	c.1061delC	<2 months	2 years	+	–	+	na; 134	–	NGT, infliximab, illeostomy, mercaptopurine, steroids	Alive 4 years
	3[na]	c.210G>T	2 months	na	+	–	+	na; 6	Recurrent airway infections, ITP, motor delay, hypoglycemic seizures, anemia of chronic diseases, osteopenia, hypogammaglobulinemia	TPN, NGT, Rapa, IgIV	Alive 8 years
Rao et al. (2007)	1[na]	Splice junction Intron 9	na	na	+ Colitis	na	+	na	Food allergies, reactive airways disease, AHA, infections	Imuran, CSA, PD, HSCT	Alive 9 years
	3[na]	c.1271G>A	na	na	+ Colitis	na	+	na	food allergies, AHA, MGN, infections	FK506, MMF, PD, HSCT	Alive 5 years
	4[na]	c.1226A>G	na	na	+ Colitis	na	–	na	AHA	TPN, FK506, rituximab, PD, alemtuzumab, HSCT	Alive 1 years
Torgerson et al. (2007), Halabi-Tawil et al. (2009), Patey-Mariaud de Serre et al. (2009), Moes et al. (2010)	IV.1[f], 6[f], 8[f], 2[na]	g.-6247_-4859del	3 weeks	na	+	–	+	950; >3000	Food allergies, cheilitis, onychodystrophy, recurrent infections, sepsis, Hp gastritis	TPN, FK506, Rapa	Alive 6 years
	IV.2[f], 7[f], 7[f], 1[na]	g.-6247_-4859del	5 weeks	na	+	–	+	2400; 365–>2000	Food allergies, cheilitis, recurrent infections, sepsis	TPN, steroids, FK506, AZA, Rapa	Alive 9 years

Lucas et al. (2007), McLucas et al. (2007)	1[f]	Exon 10[#]	<1 years	6 years	+	−	Dermatitis	na	Hypogammaglobulinemia, anemia, pneumonias, laryngeal papillomatosis, Norwegian scabies	TPN, HSCT	Alive 7 years
Burroughs et al. (2007)	1[na]	c.1271G>A	na	na	+	+	−	na	MGN	HSCT	Alive 6 years
Fuchizawa et al. (2007), Otsubo et al. (2011)	2[f], 2[f]	c.1150G>A	2 months	na	−	−	+	na	Asthma, Adrenal Insufficiency	Steroids	Alive 10 years
Fuchizawa et al. (2007)	3[f]	c.1150G>A	19 days	na	+	−	+	na	−	−	Alive 15 years
Suzuki et al. (2007)	1[na]	c.1099T>C	8 days	na	+	+	+	na	Liver dysfunction, thrombocytopenia, sepsis	na	Exitus 4 months
Taddio et al. (2007), Gambineri et al. (2008), Passerini et al. (2011b)	1, 11, 11	c.1150G>A	Neonatal	6 years	+	+	+	4900; 1494	Thyroiditis, alopecia, AHA, interstitial pneumonia	Sterois, CSA, FK506, AZA, Rapa, IgIV	Alive 16 years
Lucas et al. (2008)	1[f]	exon 10	3 months	<1 years	+	−	+	5400; na	Thrombocytopenia, Aphthous stomatitis, EBV-induced lymphoma	Rapa, Cx, VCR, PN	Alive, 2 years 6 months
Gambineri et al. (2008)	1	c.2T>C	Neonatal	Post mortem	+	+	−	803; 3910	Sepsis	MPD, CSA, FK506, IgIV	Exitus 3 months
Gambineri et al. (2008), Passerini et al. (2011b)	3, 3	c.210+2T>G	Neonatal	6 months	+	+	+	2187; na	Hypothyroidism, hepatitis, sepsis	MPD, CSA, FK506, AZA, IgIV, HSCT	Alive 5 years
Gambineri et al. (2008)	4[na]	c.543C>T	Neonatal	4 months	+	−	−	710; 3	−	MPD, CSA, IgIV	Exitus 5 months
	6[na]	c.816+5G>A	neonatal	1 years	+	+	+	na; 517	−	PD, AZA	Exitus 9 months
Gambineri et al. (2008), Passerini et al. (2011b)	7[na], 7[na]	c.967+4A>G	Neonatal	<1 years	+	+	+	700; >2000	Hepatitis	MPD, FK506, AZA	Alive 9 years

(Continued)

Table 1 | Continued

Reported by	Pt°	Mutation	Age at onset	Age at diagnosis	Diarrhea	T1DM	Eczema	Eos (/mm^3); IgE (IU/mL)	Additional clinical findings	Therapy	Outcome♦
Gambineri et al. (2008), Passerini et al. (2011b)	8[na], 8[na]	c.1015C>G	Neonatal	5 months	+	+	−	na	AIH, AHA, hepatosplenomegaly	MPD, FK506, AZA, Rapa	Exitus 6 months
Gambineri et al. (2008), McMurchy et al. (2010), Passerini et al. (2011a,b)	9, 9, 9	c.1040G>T	Neonatal	1 years	+	+	+	498; 1966	AIH, AIT, anemia, food allergy	PD, CSA	Alive 15 years
Gambineri et al. (2008), McMurchy et al. (2010)	10[na], 10[na]	c.1040G>T	<1 year	na	Severe chronic gastritis	+	Xerosis	na; >230	Pancreatic exocrine failure, gastrectomy	MPD, CSA	Alive 23 years
Gambineri et al. (2008)	13[na]	c.1121T>G	Neonatal	<1 year	+	−	+	na; 7000	Alopecia, AHA, AIT, CMV infection	MPD, FK506, AZA	Exitus, 11 months
Gambineri et al. (2008), Passerini et al. (2011a,b)	14[na]	c.725T>C	4 months	11 years	+	−	+	1550; 5218	Sepsis, nephropathy	MPD, PD, CSA, FK506	Alive 15 years
Costa-Carvalho et al. (2008)	1[f]	c.1045-3C>G	Birth	na	+	+	+	N; na	Hypothyroidism, AHA, infections	na	Exitus, 11 months
Yong et al. (2008)	1	c.1061delC	2.5 years	<5 years	+	−	Dermatitis	na	Infections	Steroids, mesalazine, infliximab, AZA, 6-MP, rapa	Alive 7 years
	2[f]	c.210G>T	1 week	7 years	+	−	+	na	Respiratory and GI infections, AHA, ITP	IgIV, TPN, steroids, rapa	Alive 8 years
Zhan et al. (2008)	1[na]	c.1139C>T	4 months	5 months	+	−	−	na; high	–	PDN, AZA, FK506, TPN, HSCT	Alive 3 years
Redding et al. (2009)	1[f]	c.1150G>A	6 weeks	na	+	−	+	3753; 157	External otitis, sepsis, bacteremia, AHA	CSA, PDN, HSCT	Alive 2 years

Halabi-Tawil et al. (2009)	1[na]	c.1113T>G	na	na	+	e	+ Erythro-derm	na	Congenital ichthyosis, HA, recurrent infections, sepsis	na	na
	2[na]	c.736-1G>A	na	na	+	e	+ Erythro-derm	na	Cheilitis, HA, MGN, recurrent infections, sepsis	na	na
	3[na]	c.1101C>G	na	na	+	e	+	na	Recurrent infections, sepsis	na	na
	4[na]	c.560C>T	na	na	+	e	+ Psori-asiform rash	na	Cheilitis, onychodystrophy, HA, recurrent infections	na	na
	5[na]	c.1121T>G	na	na	+	e	+ Psori-asiform rash	na	HA, MGN, recurrent infections, sepsis	na	na
	8[na]	c.751_753 delGAG	na	na	+	e	−	na	HA, recurrent infections	na	na
	9[na]	c.751_753 delGAG	na	na	+	e	−	na	HA, recurrent infections, sepsis	na	na
D'Hennezel et al. (2009)	1	c.1150G>A	birth	<7 weeks	+	+	Exfoliative der-matitis	na	Hypothyroidism, Respiratory Distress, Seizures, Renal Failure, Pancytopenia	TPN, rapa	Exitus 7 weeks
Patey-Mariaud de Serre et al. (2009)	1[na]	Truncated Protein	1.5 months	na	+	−	Dermatitis	na; N	AIT	na	na
	2[na]	truncated protein	6.5 years	na	+	−	Dermatitis	na; N	Allergic Asthma	na	na
	3[na]	c.1100T>G	1 year	na	+	+	−	na; N	tubulointerstitial nephritis	na	na
	4[na]	p.E251del	4 months	na	+	+	−	na; high	AHA, AIN	na	na
	5[na]	c.1121T>G	2 months	na	+	+	Dermatitis	na; high	AIT, AIN	na	na
	6[na]	c.1113T>G	4 months	na	+	−	Dermatitis	na; N	AHA, AIT	na	na
	9[na]	c.560C>T	11 months	na	+	−	Dermatitis	na; high	AIT, food allergy	na	na
	10[na]	p.E251del	7 months	na	+	+	−	na; high	AIT, AIN, tubulointerstitial nephritis	na	na
	11[na]	Truncated protein	6 months	na	+	+	Dermatitis	na; N	AHA, AIT, MGN	na	na
	12[na]	p.E251del	1 year	na	+	−	−	na; N	–	na	na

(Continued)

Table 1 | Continued

Reported by	Pt°	Mutation	Age at onset	Age at diagnosis	Diarrhea	T1DM	Eczema	Eos (/mm^3); IgE (IU/mL)	Additional clinical findings	Therapy	Outcome♦
Hashimura et al. (2009), Otsubo et al. (2011)	1[f], 4[f]	c.748_750 delAAG	2 months	5 years	− Vomiting	+	+	na; 1141	Food allergy, nephrotic syndrome, infections, AHA, sepsis	CSA, MPD	Alive 5 years
Rubio-Cabezas et al. (2009)	I	c.1222G>A	2 days	na	−	+	−	na, N	Nephrotic syndrome, TIA, chronic diabetes complications	na	Alive 15 years
	IIa[f]	c.1222G>A	3 weeks	na	+	+	−	na; N	Thyroiditis, mucocutaneous candidiasis, infections	na	Alive 12 years
	IIb[f]	c.1222G>A	3.5 months	na	+	+	−	na; N	Thyroiditis, mucocutaneous candidiasis, infections	na	Alive 12 years
	III	c.1010G>A	30 days	na	+	+	−	na; 2266	–	na	Exitus, 13 months
	IV	c.1015C>G	1 week	na	Mal digestion	+	+	na; N	Thyroiditis	na	Exitus, 5.5 months
	V	c.227delT	1 day	na	+	+	−	−; 132	Anemia, neutropenia, thrombocytopenia, dysthyroidism, infections	na	Exitus 8 months
Scaillon et al. (2009), McMurchy et al. (2010)	1[na]	c.1040G>A	8 months	19 years	Gastritis	+	−	N; N	Autoimmune gastritis, pancreatic atrophy, hypo-γ-globulinemia, infections, bronchiectasis,	PDN	Alive 22 years
Dorsey et al. (2009)	1[f]	c.***878A>G	neonatal	4.5 months	+	[§]	+	850; >5000	Sepsis	Rapa, MTX, PD, HSCT	Alive 1 years
Burroughs et al. (2010)	1[na]	c.210+2delT	2 months	2 months	+	+	−	1000–2000; 183	Hemolytic anemia, infections	HSCT	Alive, 4 years 9 months
	2[na]	c.816+7G>C	na	11 years	+	+	−	2000; 842	Anemia, steroid-dependent interstitial lung disease, membranous nephropathy, hypothyroidism, infections	HSCT	Alive 17 years

Harbuz et al. (2010)	F1[f] – II3, II4, IV4, IV5, 3, 4, 5, 6	c.816 + 4A>G[#]	na	–	6/6	na	na	na	sepsis	PN	Exitus <5 years 6/6
	F2[f] – 1	c.816 + 4A>G	2 months	Post mortem	+ Vomiting	–	+	na; >4200	Sepsis	Steroids, TPN	Exitus 3 years
Moes et al. (2010)	3[na]	g.560C>T	Birth	na	+	–	Skin pathol,	High; 5500	Thrombocytopenia, Basedow hyperthyroidism, Hp gastritis, allergy	FK506, HSCT	Exitus 8 years
	4[na]	c.1121T>G	Birth	na	+	–	+	High; 8500	Hemolytic anemia, thrombocytopenia, allergy	FK506, Rapa	Exitus, 14 months
	5[na]	c.751_753 delGAG	6 weeks	na	+	–	+	High; 12,500	Hypothyroidism, interstitial nephritis, hemolytic anemia,	FK506, Rapa, HSCT	Exitus 10 years
	6[na]	c.751_753 delGAG	4 weeks	na	+	+	Skin pathol	na; 2150	AIH, hemolytic anemia, agranulocytosis	FK506	Exitus 8 months
	7[na]	c.1015C>G	7 days	na	+	+	Skin pathol, no eczema	na; 650	Hemolytic anemia	FK506	Exitus 7 months
Tsuda et al. (2010)	1	c.210 + 1G>A	na	na	+	+	+	na; 3700	Thyroiditis, hepatitis, nephropathy	HSCT	na
	2	c.210 + 1G>A	na	na	–	–	+	na; 3210	nephropathy	na	na
	3	c.543C>T	na	na	+	–	–	na; 1	–	na	na
	4	c.816 + 7G>C	na	na	+	+	+	na; 842	Thyroiditis, nephropathy, recurrent infections	na	na
	5	c.817G>T	na	na	+	–	+	na; 364	Thyroiditis	na	na
	8	c.1150G>A	na	na	+	–	+	na; 2444	–	na	na
	9	c.1157 G>A	na	na	+	–	–	na	–	na	na
	10	c.1169G>A	na	na	+	+	+	na; 2950	Recurrent infections	na	na
	11	c.1190G>A	na	na	+	+	+	na; 657	–	na	na
	12	c.***876A>G	na	na	+	–	+	na	–	na	na
Wang et al. (2010)	1[na]	Intron1	2.5 months	2.5 months	+	+	+	na; +	Thrombocytopenia, hepatitis, hypothyroidism, infections	na	Exitus 4.5 months
An et al. (2011)	1[f]	c.1080_1081 insA	20 days	Post mortem	+	+	+	9910; 75	Proteinuria, Sepsis	Supportive treatment	Exitus 1 month

(Continued)

Table 1 | Continued

Reported by	Pt°	Mutation	Age at onset	Age at diagnosis	Diarrhea	T1DM	Eczema	Eos (/mm^3); IgE (IU/mL)	Additional clinical findings	Therapy	Outcome♦
	2[f]	c.1110G>A	14 days	Post mortem	+	−	+	22; 681	Nephrotic syndrome, lymphadenopathy, splenomegaly, pneumonia	Supportive treatment	Exitus 11 months
	3[f]	c.970T>C	26 days	Post mortem	+	+	−	3450; 3	Pneumonia	Supportive treatment	Exitus 5 months
Bae et al. (2011)	1	c.210 + 1G>A	11 months	11 years	+	+	−	N; na	PRCA, MGN, infections	PD	Alive 13 years
Kobayashi et al. (2011)	2	c.1-23G>T	na	na	+	+	−	na	Nephrotic syndrome	CSA, CS	Alive, age na
Otsubo et al. (2011)	5[f]	c.210 + 1G>T	6 months	na	+	+	−	na; na	Nephrotic syndrome	CSA, steroids	Alive 26 years
Kasow et al. (2011)	1[f]	c.1150G>A	1.5 months	<7 months	+	−	+	+; 157–1000	AHA, infections	Rituximab, CSA, PD, HSCT	Alive 3 years 7 months
Lopez et al. (2011)	1	c.748_750 delAAG	2 months	na	+	+	+	+; 45	AIH	PD, CSA, AZA, HSCT	Alive 6 years
Passerini et al. (2011b)	17	c.1037T>C	Neonatal	<4 months	+	−	Seborrhoeic dermatitis	467; 1278	Infections, sepsis	MPD, FK506, HSCT	Alive 3 years
	18	c.***876A>G	Neonatal	na	+	−	Seborrhoeic dermatitis	2300; >2000	Hypotonia	TPN, steroids, CSA, HSCT	Alive 8 years
Passerini et al. (2011a)	20	c.816 + 2delT	5 months	27 years	+	−	+	20; 424	AIT, osteomyelitis, arthritis, *S. aureus* sepsis, bronchitis	CSA, MPD, Rapa	Alive 28 years

Pt, patient; Eos, eosinophils; na, not available; N, within normal ranges; e, unspecified endocrinopathy; ITP, idiopathic thrombocytopenic purpura; AIT, autoimmune thrombocytopenia; AIN, autoimmune neutropenia; PRCA, pure red cells aplasia; MGN, membranous glomerulonephritis; AHA, autoimmune hemolytic anemia; HA, hematological abnormalities (cytopenias, hepatosplenomegaly, or lymphadenopathy); AIH, autoimmune hepatitis; TIA, transient ischemic attack; MSSA, Methicillin-sensitive Staphylococcus aureus; PN, parenteral nutrition; TPN, total parenteral nutrition; NGT, nasogastric tube; PD, prednisone; PDN, prednisolone; CSA, cyclosporine; FK506, tacrolimus, MTX, methotrexate; AZA, azathioprine; Cx, cyclophosphamide; VCR, vincristine; HSCT, hematopoietic stem cells transplantation; FU follow-up; TNDM, transient neonatal diabetes; GI, gastrointestinal.

**In this case, tubulonephropathy could be due both to the underlying disease or to tacrolimus.*

° Patient ID refers to the enumeration of patients as reported in the original publications listed in column 1.

§ Hypo or hyperglycemia.

[f] Positive familial history.

#The mutation has not been studied in this patient but in other relatives with an IPEX phenotype belonging to the same gender.

♦The age written in the outcome column refers to the age of the patients at the latest follow-up from each publication.

later, in two unrelated kindred with IPEX phenotype, Chatila et al. (2000) identified mutations in *JM2* (later called *FOXP3*) in the centromeric region of the X chromosome (Xq11.3-q13.3). Shortly after, Bennett et al. (2001b) and Wildin et al. (2001) confirmed that IPEX syndrome is the human equivalent of the *scurfy mouse*, the natural mouse model of the disease, and identified mutations in the *FOXP3* gene in additional IPEX patients. Of note, in the first family described in 1982, the disease mapped to the pericentromeric region of the X chromosome (Bennett et al., 2000), but no identifiable mutation on *FOXP3* was found, so that it was suspected to have a non-coding mutation that affects transcriptional regulation or RNA splicing (Bennett et al., 2001b).

The highly conserved *FOXP3* gene is composed of 12 exons encoding a protein of 431 amino acids in humans. Among the 63 mutations reported thus far (**Figure 1**), the majority of them (27/63) alter the C-terminal forkhead (FKH) DNA-binding domain of the protein, while the remaining of the mutations occur outside the FKH domain. The latter include mutations affecting the N-terminal proline-rich (PRR) domain (14/63), the leucine-zipper (LZ) domain (5/63), the LZ-FKH loop (9/63), the region upstream the initial ATG (3/63), and the C-terminal (3/63; **Figure 1**).

Moreover, mutations of the polyadenylation site of the gene (2/63) have been described, which lead to the expression of an unstable FOXP3 mRNA and usually result in severe, early onset disease (Bennett et al., 2001a; Dorsey et al., 2009; Tsuda et al., 2010; Passerini et al., 2011b). Patients with mutations that abrogate expression of functional FOXP3 protein (i.e., missense or frameshift mutations or splicing defects resulting in a premature stop codon) tend to have severe presentation as well (Gavin et al., 2006; Gambineri et al., 2008; Burroughs et al., 2010; An et al., 2011). Nonetheless, the severity of the disease is not always dependent on the absence of protein expression. The majority of affected individuals have missense mutations (usually point mutations) resulting in a normal or reduced level of expression of mutant protein. Such mutations lead to an impaired transcriptional regulatory activity by altering the binding sites to DNA, the interaction with other molecules (e.g., NFAT, AP1, RORα), or the dimerization of FOXP3 (**Figure 1**).

Independently from the type or site of the *FOXP3* mutation, all patients described but five (Ferguson et al., 2000; Fuchizawa et al., 2007; Rubio-Cabezas et al., 2009; Scaillon et al., 2009; Tsuda et al., 2010; Otsubo et al., 2011) developed gastrointestinal symptoms (mainly diarrhea).The exact nature of genotype-phenotype correlation has been difficult to pinpoint, especially considering the age at onset and the disease outcome. For example, in 13 patients presenting with the same mutation (c.1150G>A), the onset ranged from birth to 7 months (**Table 1**). In addition the outcome was influenced by other factors such as timing of the therapeutic intervention, concomitant infections, and each individual patient's response to therapy.

The histopathological lesions also differ among the patients carrying the same mutation, further suggesting that the genotype does not strictly correlate with phenotypical changes of the target organs (Patey-Mariaud de Serre et al., 2009). This inconsistent correlation between genotype and phenotype may reflect the complex intracellular interactions of FOXP3 (Allan et al., 2005) and also strongly suggests the role of environmental or epigenetic factors that might participate in determining the clinical picture and outcome (Gambineri et al., 2008).

CLINICAL MANIFESTATIONS

Most IPEX patients are born at term after an uneventful pregnancy from unrelated parents. A careful family history may reveal the presence of male subjects in the maternal lineage with similar clinical phenotype, early death, or multiple spontaneous abortions. Notably, these patients may have other affected brothers, but females belonging to the same lineage are usually healthy.

At birth, they may have a normal weight and length without pathological findings. The onset of IPEX syndrome usually occurs

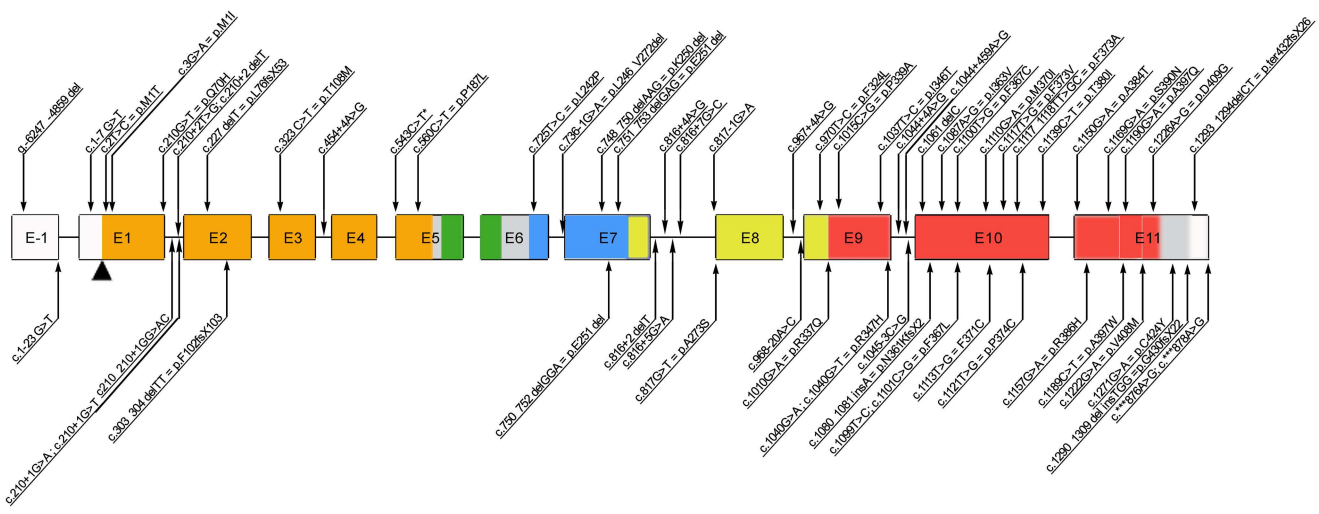

FIGURE 1 | Schematic representation of the *FOXP3* gene reporting all the mutations published so far. Annotations refer to both coding sequence and protein, when applicable (www.ncbi.nlm.nih.gov/CCDS, accession number CCDS14323.1). *c543C>T is a polymorphism. E, exon; Color code: orange, N-terminal domain; green, zinc finger domain; blue, leucin-zipper domain; red, forkhead domain.

in males within their first months of life, but in some cases even after few days or weeks, and can be rapidly fatal if not diagnosed and treated. The most severe cases are characterized by the early onset of a triad of clinical manifestations: intractable diarrhea, type-1 diabetes mellitus (T1DM), and eczema.

Autoimmune enteropathy is a hallmark of IPEX syndrome. Patients present with neonatal, watery, and sometimes mucoid or bloody acute diarrhea. This acute severe enteropathy often begins in the first days of life or during breast-feeding, thus showing to be independent from cow milk or gluten introduction in the diet. However, it could be worsen by switching from breast-feeding to regular formula. It typically persists despite dietary exclusions and bowel rest. Since it results in severe malabsorption and significant failure to thrive, parenteral nutrition is often required. In addition to diarrhea, other gastrointestinal manifestations can present, such as vomiting (Ferguson et al., 2000; Hashimura et al., 2009; Harbuz et al., 2010; Otsubo et al., 2011), gastritis (Nieves et al., 2004; Gambineri et al., 2008; Scaillon et al., 2009), ileus (Levy-Lahad and Wildin, 2001), and colitis (Lucas et al., 2007; Otsubo et al., 2011; **Table 1**).

Type-1 diabetes mellitus can precede or follow enteritis. T1DM is present in the majority of patients including newborns, and is usually difficult to control (Peake et al., 1996; Baud et al., 2001; Gambineri et al., 2008). There have been rare cases (6/136) presenting with diabetes mellitus without auto-antibodies (Rubio-Cabezas et al., 2009; Scaillon et al., 2009). Imaging studies or autopsy and histological examination often reveal destruction of the pancreas and intense lymphocytic infiltrate, suggesting that an immune mediated damage of this organ may have a role in the pathogenesis (Wildin et al., 2002; Costa-Carvalho et al., 2008; Rubio-Cabezas et al., 2009).

Cutaneous manifestations appear in the first months of life. Similar to diarrhea and diabetes, cutaneous manifestations are very common (95/136) and can be the first sign of the disease (**Table 1**).

Dermatitis can be eczematiform (mainly atopic dermatitis) (Wildin et al., 2002; Owen et al., 2003; Ruemmele et al., 2008), ichthyosiform (Baud et al., 2001; Rao et al., 2007), psoriasiform (Nieves et al., 2004; De Benedetti et al., 2006), or any combination of the above (e.g., atopic dermatitis and psoriasis coexisting on different areas of the skin) (Halabi-Tawil et al., 2009). Skin involvement is severe and diffuse, characterized by erythematous exudative plaques that could evolve into more lichenfied plaques (Halabi-Tawil et al., 2009). Pruritus can be a major complain in these patients since it is intense and difficult to control with anti-histamine drugs. Cutaneous lesions often show resistance to classic treatments such as topical steroids or tacrolimus and can be complicated by bacterial infections (most commonly *Staphylococcus aureus* and *epidermidis*) with potential development of sepsis (Halabi-Tawil et al., 2009). Other manifestations affecting the integumentary system include: painful and fissurary cheilitis (Halabi-Tawil et al., 2009), onychodystrophy (Halabi-Tawil et al., 2009), and alopecia (Nieves et al., 2004; Moudgil et al., 2007; Gambineri et al., 2008).

Two patients presented with severe *allergies* to food or other allergens causing asthma, skin rashes, and gastrointestinal symptoms in the absence of endocrinopathies. These patients were initially diagnosed and treated as severely allergic individuals (Torgerson et al., 2007). Given this, severe allergic conditions in association with other autoimmune symptoms should raise the suspicion of IPEX syndrome.

The clinical picture can be complicated by the presence of other autoimmune symptoms (**Table 1**): thyroiditis (27/136) with either hyperthyroidism or, more commonly, hypothyroidism (Kobayashi et al., 2001;Wildin et al., 2001, 2002; Nieves et al., 2004; Myers et al., 2006; Moudgil et al., 2007; Costa-Carvalho et al., 2008; Gambineri et al., 2008; Halabi-Tawil et al., 2009; Rubio-Cabezas et al., 2009; Wang et al., 2010; Otsubo et al., 2011) cytopenias (42/136) such as hemolytic anemia, thrombocytopenia, and neutropenia, and hepatitis (8/136) that may be autoimmune with positive auto-antibodies (**Table 1**). Renal disease can be related either to autoimmunity or to prolonged administration of nephrotoxic drugs. They are generally described as tubulonephropathy (Kobayashi et al., 2001; Otsubo et al., 2011) and nephrotic syndrome (Gambineri et al., 2008; Rubio-Cabezas et al., 2009; An et al., 2011; Otsubo et al., 2011), although interstitial nephritis (Bindl et al., 2005; Patey-Mariaud de Serre et al., 2009; Moes et al., 2010) and membranous glomerulonephritis (Moudgil et al., 2007; Halabi-Tawil et al., 2009; Burroughs et al., 2010; Bae et al., 2011) have also been found in some patients' histopathological examinations. A rare manifestation associated with the milder forms of IPEX with delayed diagnosis is arthritis involving one or more joints (Wildin et al., 2002; De Benedetti et al., 2006). Splenomegaly and lymphadenopathy may progress as a result of an ongoing autoimmune lymphoproliferation, as evidenced by the extensive lymphocytic infiltrates in secondary lymphoid organs found in several patients during autopsy (Wildin et al., 2002; Ochs and Torgerson, 2007; Costa-Carvalho et al., 2008). Despite multiple and early autoimmune manifestations typical of IPEX syndrome, it is important to underline that their number may increase with age. IPEX patients' presentation typically begins early with some of these autoimmune symptoms, and progresses with new manifestations over years.

The clinical spectrum can be worsened by infections, although they are less frequent than the more prominent signs described above. The onset of IPEX syndrome is often associated with infections, however a clear causative role of pathogens in the onset of autoimmunity has not been demonstrated and infections can often be the consequence of multiple immunosuppressive (IS) therapy and poor clinical conditions.

The most frequent infections are pneumonia, airway infections, gastrointestinal, and skin super-infections that may lead to life-threatening sepsis from *Enterococcus* spp. and *Staphylococcus* spp. (Halabi-Tawil et al., 2009). Other common pathogens are *Clostridium difficile*, *Candida albicans*, *Pneumocystis jiroveci*, CMV, and EBV.

LABORATORY FINDINGS

Laboratory tests can be normal at onset. There are no specific diagnostic findings in IPEX syndrome although the laboratory abnormalities consistent with T1DM and severe enteropathy are common. Moreover, other alterations may suggest ongoing autoimmune manifestations in other target organs, such as hypothyroidism, cytopenias, hepatitis, or nephropathy. Markedly elevated IgE levels and eosinophil counts are observed in the majority

of patients as an early hallmark of the disease (**Table 1**). Serum IgA, IgG, and IgM levels are generally normal or low due to the protein-losing enteropathy.

Patients in the acute phase of the disease, prior to IS therapy, can have normal or elevated white blood cell counts. Leukocytosis, if present, is due to an increase in lymphocytes but the percentage of the different lymphocyte subpopulations (CD3, CD4, CD8, CD16, CD19) remains unchanged despite immune dysregulation. The CD4/CD8 ratio is maintained or increased and the T cell repertoire is polyclonal. The percentages of naive and memory T cells are mostly comparable to their age-matched controls. The $CD4^+CD25^+FOXP3^+$ Treg cells are present (Gavin et al., 2006; Gambineri et al., 2008), but FOXP3 expression can be reduced if *FOXP3* mutation prevents the expression of the protein (Bacchetta et al., 2006) or if the patient is exposed to IS therapy (Gambineri et al., 2008). In addition, *in vitro* proliferative responses to mitogens are normal unless the patient is treated with IS drugs (Bacchetta et al., 2006). The *in vitro* cytokine production shows a decrease in Th1 cytokines and an increase in Th2 (Chatila et al., 2000; Nieves et al., 2004; Bacchetta et al., 2006). The karyotype is normal.

A variety of auto-antibodies are detected in most patients and their presence usually correlates with signs of pathology in the target organs, but their production may also be a sign of immune dysregulation without an obvious pathological linkage (Tsuda et al., 2010).

There is increasing evidence that anti-enterocyte antibodies are characteristic of IPEX patients, although not all patients have been tested because the assay is not widely accessible. The autoimmune enteropathy-related 75 kDa antigen (AIE-75), predominantly expressed in brush border of the small intestine and proximal tubules of the kidney, has been identified as a specific target of the auto-antibodies present in IPEX patients sera (Kobayashi et al., 1998, 1999, 2011; Gambineri et al., 2003; Patey-Mariaud de Serre et al., 2009; Moes et al., 2010).

In addition, a recent study of Kobayashi et al. identified villin, a 95-kDa actin-binding protein, as another brush border antigen aberrantly targeted in IPEX syndrome. Like AIE-75, villin is also expressed both in the microvilli of the small intestine and in the proximal renal tubules. In this study, five out of five IPEX patients showed anti-AIE-75 antibodies and four out of five displayed anti-villin antibodies. None of the control sera from healthy subjects or patients affected by non-IPEX pathologies (e.g., autoimmune enteropathies of different origin, enterocolitis, and colon cancer) were positive for anti-AIE-75 antibodies and only a few were weakly positive for anti-villin antibodies. High levels of anti-villin auto-antibodies have been found only in children with IPEX syndrome (Kobayashi et al., 2011). These findings confirm the specificity of both anti-AIE-75 and anti-villin antibodies for IPEX syndrome. Their link to the tissue damage, the correlation to the progression of the disease, and their predictive value have to be clarified.

Early presence of detectable auto-antibodies against insulin, pancreatic islet cells, or anti-glutamate decarboxylase correlates with occurrence of neonatal T1DM. Moreover, anti-thyroglobulin and anti-microsome peroxidase antibodies are detected in autoimmune thyroiditis even in the absence of functional impairment; Coombs antibodies, anti-platelets antibodies, and anti-neutrophils antibodies are often present in autoimmune cytopenias; anti-smooth muscle (ASMA) and anti-liver-kidney-muscle (anti-LKM) antibodies are positive in autoimmune hepatitis. Recently, Huter et al. (2010) reported that sera from IPEX patients react against keratins, especially keratin 14, suggesting this molecule as a target for autoreactive lymphocytes in the skin of IPEX patients.

Although there is no pathognomonic finding specific to IPEX, biopsies of the affected organs can help in excluding other etiologies. Main histological findings in the gastrointestinal tracts are total or subtotal villous atrophy with mucosal lymphocytic and eosinophil infiltration, but they are not specific for the disease. In a recent work, Patey-Mariaud de Serre and colleagues described the intestinal morphological changes of twelve IPEX patients (Patey-Mariaud de Serre et al., 2009). Three different kinds of lesions were found in the gastrointestinal tract: (1) the graft-versus-host disease-like pattern was the most frequent form observed; (2) the celiac disease-like pattern, found in two patients; (3) depletion of the intestinal goblet cells along with the presence of anti-goblet cell auto-antibodies, reported in one child. Hence, one of these histopathological patterns in the proper clinical context and an association with circulating anti-AIE-75 auto-antibodies would suggest the diagnosis of IPEX syndrome.

In addition, one case reported the autoimmune destruction of pancreatic exocrine cells contributing to the diarrheal disease (Heltzer et al., 2007).

The histopathological changes at the skin biopsies are usually non-specific for IPEX syndrome since there is a wide range of possible dermatological pictures. The clinical and histopathological features of skin pathology of 10 IPEX patients were described by Halabi-Tawil et al. (2009). Either subacute /chronic spongiotic dermatitis or psoriasiform changes, also consistent with a chronic lichenified eczema, have been shown. One out of the four biopsies showed a slight perivascular lymphocytic infiltrate in the upper dermis, while the others showed a moderate to intense superficial dermal infiltrate with the simultaneous presence of eosinophil and lymphocyte infiltrates. Although the majority of skin alterations were compatible with atopic or psoriasiform dermatitis, IPEX patients may present with uncommon allergic (Nieves et al., 2004), autoimmune (Ferguson et al., 2000; McGinness et al., 2006), or infectious (McLucas et al., 2007) dermatological complications.

DIFFERENTIAL DIAGNOSIS

A neonate presenting a single severe manifestation of IPEX syndrome such as enteropathy, diabetes, or newborn erythroderma may pose a diagnostic challenge for the physician. For each of them, the suspicion of IPEX syndrome should be raised once other more common diseases have been excluded.

In a neonate presenting with isolated diarrhea, an autoimmune pathogenesis of the enteropathy is a rare event. **Table 2** provides a summary of the possible causes of enteropathy in newborns and infants. IPEX enteropathy, like other diarrheal diseases, may have either an aggressive or insidious onset. When the onset of the diarrhea is acute, microbial origins need to be excluded first. When the diarrhea persists, a wide range of differential diagnosis has to be considered (Murch, 2001). The most common cause is

Table 2 | Differential diagnosis of early onset persistent diarrhea.

Infectious and post-enteritis diarrhea
FOOD-SENSITIVE ENTEROPATHY OR ENTEROCOLITIS
Cow's milk sensitive enteropathy (most frequent)
Celiac disease
Non-celiac gluten sensitivity
Food protein induced enterocolitis
Eosinophilic gastroenteropathy
ANATOMICAL DEFECTS AND DYSMOTILITY DISORDERS
Hirschsprung disease
Intestinal lymphangiectasia
Short bowel syndrome (post surgery)
Stagnant loop syndrome (post surgery)
Chronic intestinal pseudo-obstruction
TRANSPORT DEFECTS
Chloride-bicarbonate exchanger defect (chloride-losing diarrhea)
Sodium hydrogen exchanger (congenital sodium diarrhea)
Ileal bile acid receptor defect
Sodium-glucose cotransporter defect (glucose-galactose malabsorption)
Abetalipoproteinemia
Hypolipoproteinemia
Acrodermatitis enteropathica (zinc deficiency)
ENZYMATIC DEFECTS
Enterokinase deficiency
Disaccharidase congenital defect (lactase, sucrase-isomaltase)
PANCREATIC MALABSORPTION
Cystic fibrosis
Shwachman syndrome
PRIMARY EPITHELIAL CAUSES OF INTRACTABLE DIARRHEA
Microvillous inclusion disease
Tufting enteropathy
Heparan sulfate deficiency
IMMUNODEFICIENCIES (USUALLY UNMASKED BY A PATHOGEN)
Severe combined immunodeficiency (SCID)
Thymic hypoplasia
Class II major histocompatibility (MHCII) deficiency
CD40 ligand deficiency
Neutrophilic specific granule defect
Acquired immunodeficiency syndrome (AIDS)
IBD (very rare in infancy, to be considered as a part of a PID)
METABOLIC DISEASES
Mitochondrial myopathy
Wolman disease
AUTOIMMUNE ENTEROPATHY

food-sensitive enteropathy, so appropriate exclusion diets should be initiated for an adequate period. Anatomical abnormalities such as malrotation and pseudo-obstruction may cause bacterial overgrowth with chronic diarrhea and malabsorption. If chronic diarrhea is associated to protein-losing enteropathy, lymphangectasia should also be considered. Transport or enzyme disorders induce selective malabsorption of glucose-galactose, lipids, fat-soluble vitamins, amino acids, electrolytes, and zinc (Murch, 2001, 2006). In some of these cases, diarrhea would be abrogated by withdrawing oral feeding. Moreover, malabsorption could be in some cases related to pancreatic disease rather than to an intestinal transport or enzymatic alteration. Nevertheless, the intestinal biopsies in both cases show a normal architecture with intact villous-crypt axis, unlike in IPEX. On the contrary, primary epithelial enteropathies, such as microvillous inclusion disease and tufting enteropathy, are characterized by blunting villi at the intestinal biopsy and usually appear in the first days after birth. They should be excluded if diarrhea is prolonged and continues during total parenteral nutrition (Sherman et al., 2004). Immunodeficiencies, such as severe combined immunodeficiency (SCID) or intermediate forms of combined immunodeficiency (CID), may present first with gastrointestinal symptoms, often fatal in early childhood if untreated (Geha et al., 2007). In the latter cases, diarrhea may be due to a prolonged impairment to clear enteric pathogens or to a primary concomitant autoimmunity. Even metabolic diseases or endocrinopathies could manifest with chronic diarrhea. Further metabolic and hormonal assessment should be considered in such cases. Autoimmune enteropathy is usually a diagnosis of exclusion. Once the aforementioned diseases have been excluded by appropriate clinical or laboratory evaluations, the presence of the following clinical and histological findings indicative of the autoimmune pathogenesis, should be considered: an unresponsiveness to dietary restriction and total parenteral nutrition, an association with other autoimmune conditions (Unsworth and Walker-Smith, 1985), small intestinal villous atrophy with hyperplastic crypt, mononuclear cells infiltrate within the intestinal mucosa (Murch, 1997). Autoimmune enteropathy can be also one of the symptoms of complex forms of immune dysregulation, but other clinical or laboratory features usually help to distinguish them from IPEX syndrome (**Table 4**).

The onset of permanent diabetes mellitus in the neonatal age is described as a rare event (Rubio-Cabezas et al., 2010). Although autoimmune T1DM is diagnosed in over 95% of children presenting with diabetes after 6 months of age (Porter and Barrett, 2004), alternative etiologies should be considered in newborns and young infants presenting with diabetes before 6 months of age (Hattersley et al., 2009). Most of these patients have a monogenic form of disease, even if the responsible gene remains unknown in up to 40% of patients (Edghill et al., 2008). The main monogenic causes of early onset diabetes are mutations in Kir6.2 gene (the inward rectifier subunit of the ATP-sensitive potassium channel of the β cells), in SUR1 gene (the regulatory subunit of the K_{ATP} channel in pancreatic β cells) and in the preproinsulin gene. Mutations of chromosome 6q24 and mutations of the insulin gene may also be considered (Valamparampil et al., 2009; Greeley et al., 2010). The presence of auto-antibodies specific for pancreatic antigens before 6 months of age should however pose the question of *FOXP3* mutation (Greeley et al., 2010). A recent study reported that 4% of male patients with permanent neonatal diabetes were found to have *FOXP3* mutations (Rubio-Cabezas et al., 2009). The diagnosis of IPEX becomes more obvious when diabetes is preceded or followed by other symptoms related to immune dysregulation, such as enteropathy and eczema.

Skin pathology is a common finding in infants diagnosed with IPEX syndrome. The absence of other clinical signs may delay

Table 3 | Differential diagnosis of erythroderma presenting in the neonatal period.

INFECTIONS
Staphylococcal scalded skin syndrome (SSSS)
Congenital cutaneous candidiasis
IMMUNODEFICIENCY
Graft-versus-host disease (GvHD) with underlying SCID
Omenn's syndrome
ICHTHYOSES
Non-syndromic ichthyoses (non-bollous ichthyoses, bollous ichthyoses)
Syndromic ichthyoses (Netherton's syndrome, Conradi–Hünermann syndrome)
METABOLIC DISEASES
Multiple carboxylase deficiencies
Essential fatty acid deficiency
DRUGS
Ceftriaxone
Vancomycin
OTHER SKIN PATHOLOGIES
Infantile seborrheic dermatitis
Atopic dermatitis
Psoriasis
Cutaneous mastocytosis

the diagnosis, especially in neonates and infants (Nieves et al., 2004). The presentation ranges from mild eczema to severe generalized erythroderma or other unusual skin manifestations with poor response to steroids (Halabi-Tawil et al., 2009; Redding et al., 2009). Focusing on the neonates and infants presenting erythroderma as single diffuse manifestation of IPEX syndrome at onset, **Table 3** summarizes the possible clinical pictures that should be considered for differential diagnosis (Hoeger and Harper, 1998; Fraitag and Bodemer, 2010). Erythroderma is an inflammatory skin disorder affecting the majority of the body surface, with subacute or chronic evolution accompanied by scaling skin. In the neonatal period, it can also be the primary manifestation of several conditions. Perinatal or neonatal infections such as Staphylococcal scalded skin syndrome (SSSS) and congenital cutaneous candidiasis may result in diffuse skin involvement. Skin swab and/or skin biopsy is usually diagnostic.

Immunodeficiencies may present with extended skin alterations as a result of the immune aggression sustained by autoreactive newborn's lymphocytes (as in Omenn's syndrome) or maternal lymphocytes expanding after birth in the immunodeficient host (graft-versus-host disease with underlying SCID). Immunological assessment confirms the diagnosis of PID in these cases (**Table 4**). If ichthyoses is suspected, skin biopsy is diagnostic. Metabolic disorders can be associated with erythroderma, but usually it is not the only complain and other systemic signs can support the diagnosis. Ceftriaxone or Vancomycin, if recently administered, should be stopped immediately to rule out drug-induced skin reactions. Other common skin pathology of infancy, e.g., atopic eczema and psoriasis, may evolve into erythroderma, but the early presentation, the persistency of the lesions, and the limited response to topical treatment may increase the suspicion of IPEX syndrome. As recently pointed out by Leclerc-Mercier et al. (2010), early skin biopsy has a central role in excluding the majority of these pathological conditions.

The clinical characteristics that are common in PID with autoimmunity and unique to IPEX are summarized in **Table 4**. The differential diagnoses with primary immunodeficiencies associated with immune dysregulation and subsequent autoimmune phenomena, such as CD25 deficiency, STAT5b deficiency, Omenn's syndrome, Wiskott–Aldrich syndrome, Hyper IgE syndrome, autoimmune lymphoproliferative syndrome, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, should always be considered.

FOXP3 DYSFUNCTION AND DISEASE PATHOGENESIS

Forkhead box p3 is a transcription factor, master regulator for the function of thymic-derived regulatory T (nTreg) cells (Wildin et al., 2001; Fontenot et al., 2003; Bacchetta et al., 2007; Gambineri et al., 2008). These cells are among the main subsets of $CD4^+$ T cells appointed to maintain peripheral self-tolerance.

$CD4^+CD25^+FOXP3^+$ T cells can be present in normal percentage in the peripheral blood of the IPEX patients. This was demonstrated not only by immunophenotype, but also by analysis of the Treg-cell-specific-demethylated-region (TSDR; Passerini et al., 2011b; Barzaghi et al., 2012), whose demethylation ensures cell-specific stable expression of FOXP3 (Baron et al., 2007; Wieczorek et al., 2009).Therefore, in IPEX patients *FOXP3*mut Treg cells are physically present but functionally impaired, and this is considered the primary direct cause of autoimmunity in IPEX (Bacchetta et al., 2006; D'Hennezel et al., 2009; Moes et al., 2010). In this respect, IPEX syndrome is the best example of monogenic autoimmune disease due to Treg deficiency. However, autoimmunity in other immunodeficiencies, such as ADA-SCID and WAS, has been recently associated with altered function of Treg cells, regardless of FOXP3 expression (Marangoni et al., 2007; Sauer et al., 2012).

Despite the general consensus on the fact that FOXP3 is fundamental for acquisition and maintenance of suppressive function by nTreg cells (Gavin et al., 2007; Wan and Flavell, 2007; Williams and Rudensky, 2007), it is unclear how the different mutations affect their function. Functional *in vitro* studies on Treg cells of IPEX patients revealed that the degree of functional impairment of the suppressive activity varies among the patients, with complete abrogation of suppressive function in patients with null mutations (Bacchetta et al., 2006). Similarly, mutations in the FKH DNA-binding domain of FOXP3 that caused severe IPEX (p.R347H and p.F373A) were only partially blocked in their ability to reprogram conventional T cells into Treg cells (McMurchy et al., 2010). It may therefore be hypothesized that some mutated forms of the protein retain residual protein activities, thus only partially impairing FOXP3 functions. The molecular mechanisms of Treg-mediated suppression remain controversial, hence our understanding of the impact of different *FOXP3* mutations on Treg cell function is incomplete.

In addition to the well-accepted loss of suppressive function, we recently described that *FOXP3* mutations cause high instability of the Treg cell compartment, with a marked shift to the Th17 cell phenotype of *bona fide* nTreg cells expressing a mutated

Table 4 | Differential diagnosis of primary immunodeficiencies (PID) presenting with autoimmunity.

	IPEX	CD25 def	STAT5b def	OMENN'S	WAS	HIES	ALPS	APECED
Onset	Neonatal, 1 year	<1 year, early infancy	<1 year, infancy	Neonatal, 1 year	1 year, early infancy	Neonatal, 1 year	Neonatal, 1 year	Infancy, young adulthood
Enteropathy	Always	Frequent	Frequent	Frequent	Possible	Not present	Not frequent	Not frequent
Endocrinopathy	T1DM ± thyroiditis	Thyroiditis	GH unresponsiveness	Absent	Absent	Absent	Absent	Hypoparathyroidism, adrenal insufficiency ± T1DM, thyroiditis
Skin lesions	Eczema, erythroderma	Eczema, erythroderma	Eczema	Erythroderma, alopecia	Eczema	Newborn rash, eczema	Urticarial rash	Alopecia, vitiligo
Infections	Rare/secondary to IS	Recurrent/ persistent (particularly CMV)	Recurrent (viral), severe varicella	Severe	Frequent	Recurrent pulmonary, cutaneous (*S. aureus*)	Not frequent	Candidiasis
Anemia	Possible	Possible	Rare	Frequent	Possible	Absent	Frequent	Rare
Thrombocytopenia	Possible	Possible	Rare	Possible	Always	Absent	Frequent	Rare
Neutropenia	Possible	Possible	Rare	Rare	Rare	Rare	Frequent	Rare
Others	Failure to thrive, hepatosplenomegaly, lymphoadenopathy, other autoimmune manifestations	Failure to thrive, hepatosplenomegaly, lymphoadenopathy	Growth failure, chronic lung disease, interstitial pneumonia	Failure to thrive, hepatosplenomegaly, lymphoadenopathy, inflammatory pneumonitis and enteritis	Failure to thrive, hemorragies, other autoimmune manifestations, tumors	Characteristic face, cathedral palate, bone fractures	Hepatosplenomegaly, lymphadenopathy, other autoimmune manifestations	Ovarian or testicular failure, gastritis, hepatitis, keratoconjunctivitis
Eosinophilia/hyper IgE	Present	Present	Present	Present	Present	Present	Absent	Absent
Hereditary pattern	X-linked	AR	AR	AR/unknown	X-linked	AD/AR/ unknown	AD/unknown	AR
Gene	FOXP3	IL-2RA (CD25)	STAT5b	RAG1/2 (90%), Artemis/IL7RA/, ADA/DNAligase IV/γc/unknown	WASP	STAT3/TYK2/ DOCK8/ unknown	FAS/FASL/CASP8/ CASP10/unknown	AIRE

WAS, Wiskott–Aldrich syndrome; HIES, hyper IgE syndrome; ALPS, autoimmune lymphoproliferative syndrome; APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy; def, deficiency; IS, immunosuppression; T1DM, type-1 diabetes mellitus; AR, autosomal recessive; AD, autosomal dominant.

form of FOXP3 (Passerini et al., 2011b). Indeed, the plasticity between different $CD4^+$ T cell subsets is a new and dynamic concept, particularly pronounced between the Th17 and Treg cell compartments (Lee et al., 2009), although the *in vivo* relevance of such phenomenon is controversial (Zhou et al., 2009; Rubtsov et al., 2010). Thus, in addition to the loss of suppressive function, *FOXP3* mutations are associated with inflammation-driven conversion from a regulatory to an effector (i.e., IL-17-producing) phenotype of mutated Treg cells, which may directly contribute to the autoimmune damage in the target organs.

While the necessity of FOXP3 for suppressive function of Treg cells is undisputed, it is unclear whether functional FOXP3 is essential for thymic development of Treg cells in humans. Data from murine models of *FOXP3* deficiency indicate that FOXP3 is dispensable for thymic development of Treg cells, but rather essential for their maintenance in the periphery, as demonstrated in *Foxp3*^{gfpko} female mice (Gavin et al., 2007) and in FILIG mice, which display reduced Foxp3 expression in Treg cells (Wan and Flavell, 2007). On the other hand, data from healthy carriers of *FOXP3* mutations and transplanted IPEX patients with low peripheral donor chimerism clearly indicate that only Treg cells expressing a wild type FOXP3 survive long term in the periphery, although leave it unclear whether the selective advantage is already active during thymic differentiation or occurs later on in life (Di Nunzio et al., 2009; Seidel et al., 2009). Our recent observation that *bona fide* Treg cells can be detected by TSDR demethylation analysis in the peripheral blood of IPEX patients both at the onset of the disease and several years after IS treatment, regardless of FOXP3 expression, demonstrates that functional FOXP3 is not necessary for thymic differentiation of Treg cells in humans, as previously demonstrated for murine Treg cells (Gavin et al., 2007), and that FOXP3mut Treg cells can survive and be detected long term, in the peripheral blood of patients with IPEX syndrome (Passerini et al., 2011b; Barzaghi et al., 2012).

Evidences from studies on human and murine models show that Type-1 regulatory T (Tr1) cells can contribute to suppressing the development of autoimmunity in addition to nTreg cells (Roncarolo et al., 2006; Sakaguchi, 2006). We recently demonstrated that Tr1 cells can develop in IPEX patients regardless of FOXP3 expression (Passerini et al., 2011a). This observation suggests that FOXP3-independent immune regulation can potentially contribute to controlling the disease, although Tr1 cells alone do not seem adequate to suppress the initial acute phase of the disease. Thus, it is tempting to conclude that FOXP3 is not necessary for function and development of adaptive Treg cells, the IL-10 producing Tr1 cells.

In humans, FOXP3 is also expressed transiently upon activation, in conventional Teff cells (Allan et al., 2007; Tran et al., 2007; Passerini et al., 2008), in which a still unknown function has been postulated (Ziegler, 2006; McMurchy et al., 2010). This implies that *FOXP3* mutations may also impinge on Teff cell function and suggests that FOXP3-dependent Teff impaired function may directly contribute to the pathogenic mechanism underlying the disease. In support of this hypothesis are the data demonstrating an impaired Th1 cytokine production from IPEX T cells, with relative increase of Th2 cytokines (Chatila et al., 2000; Nieves et al., 2004; Bacchetta et al., 2006). In addition, we observed an increased proportion of IL-17 producing cells in the patients' PBMC, which could be derived in part from converted Treg, as mentioned above, or in part from Teff cells.

Overall, our current view of the pathogenesis of IPEX syndrome is that, even if impairment of Treg function is the major step, other factors such as inflammation and Th17 elevation can cooperate in maintaining and perpetuating the immune-dysfunction.

THERAPY

Due to the limited and sporadic number of cases reported in literature, it has been difficult up to now to compare different therapeutic strategies and relative outcomes. Therefore, the therapeutic approaches for the treatment of IPEX patients are still based on the experiences in single patients. Moreover, given the unclear genotype-phenotype correlation, the clinical course of the disease and the response to therapy can be variable and not always satisfying. Therapy is therefore targeted to the clinical manifestations and severity of the individual patient. The current treatments available for IPEX syndrome include replacement and supportive therapy, IS therapy, and hematopoietic stem cell transplantation (HSCT). Nutritional support and IS therapy should be promptly started to counteract the initial acute manifestations. A wasting syndrome can acutely affect the outcome of these patients, calling for a collaborative multi-disciplinary effort among clinicians from different specialties such as gastroenterology, infectious disease, and immunohematology.

REPLACEMENT AND SUPPORT THERAPY

At onset, the patient should be hospitalized and receive a broad-spectrum supportive care (fluids, TPN, albumin) with replacement therapy for endocrine disorders (e.g., insulin and/or thyroid hormones), autoimmune cytopenias (e.g., hemocomponents), or hypogammaglobulinemia (e.g., intravenous immunoglobulins). Prophylactic antibiotics should be used considering the multiple potential sources of infection such as skin lesion, damaged gastrointestinal lining, and central venous catheter. Infectious episodes can drastically exacerbate or complicate the existing clinical symptoms, endangering the patient's life.

IMMUNOSUPPRESSIVE THERAPY

Monotherapy or combination immunosuppression reported so far has shown to be only partially effective in controlling the autoimmune manifestations. Multiple IS therapies are often required to control symptoms (Gambineri et al., 2008).

Glucocorticoids (prednisone and methylprednisolone) are used as the first line therapy to limit progression of organ damage (Gambineri et al., 2008). If the response to prednisone is inadequate, betamethasone (the equivalent oral dose) could have significantly better efficacy (Kobayashi et al., 2001; Taddio et al., 2007). Then other IS drugs can be added onto the steroids regimen. Cyclosporine and/or tacrolimus have been most commonly used in conjunction with steroids (Baud et al., 2001; Wildin et al., 2002; Mazzolari et al., 2005; Taddio et al., 2007; Gambineri et al., 2008). Azathioprine also has been used with steroids and/or tacrolimus with partial control of the disease (Bindl et al., 2005). The ideal dose of medication should be determined to maximize clinical benefit of the individual patient while minimizing side effects.

Thanks to a better understand the disease pathogenesis, clinicians nowadays tend to choose more specific IS drugs, based on the medication's mechanism of action. Calcineurin inhibitors have partial efficacy with high toxicity and simultaneously suppress Teff cells, expression of FOXP3, and Treg cell function. On the contrary, rapamycin selectively target Teff cells and do not interfere with the function of Treg cells, which are insensitive to mTOR inhibitors (Battaglia et al., 2006; Allan et al., 2008). Even if it is not clear if FOXP3mut Treg cells respond to rapamycin in the same way as FOXP3wt, the use of rapamycin (alone or in combination with azathioprine or steroids) has given promising clinical results in four IPEX cases (Bindl et al., 2005; Gambineri et al., 2008; Yong et al., 2008). In these reports, rapamycin was used not as a first line therapy, but as a second choice when calcineurin inhibitor failed. The dosage used (approximately 0.15 mg/kg/day) was adjusted to maintain serum levels between 8 and 12 ng/mL. In three patients with IPEX syndrome, the combination of rapamycin, methotrexate, and steroid (in one case) and rapamycin, steroid, and azathioprine (in the other two) allowed to obtain clinical remission in all cases and maintain it over time (follow-up of 5 years, 6 months, and 1.5 years, respectively; Bindl et al., 2005). The same positive effect was achieved in one patient with rapamycin and steroid, and with rapamycin monotherapy in another. Both showed clinical remission with a follow-up of 21 and 15 months, respectively (Yong et al., 2008). Based on these positive responses to rapamycin, its use as the first line IS drug in conjunction with steroid might be considered instead of calcineurin inhibitors. Of note, administration of rapamycin should be accompanied by frequent monitoring of serum drug level with appropriate dose adjustment, since the enteropathy may affect the drug intestinal absorption.

In IPEX patients who survived the first years of life, immunosuppression may stabilize the existing symptoms, but flares of the disease may occur and new symptoms may arise despite the therapy.

HEMATOPOIETIC STEM CELL TRANSPLANTATION

Currently, the only cure for IPEX syndrome is allogeneic HSCT. A summary of the published data regarding HSCT in IPEX patients is provided in **Table 5**. Early HSCT leads to the best outcome, as the organs are yet to be damaged from autoimmunity and the adverse effects of therapy. For this reason it is fundamental to ensure an early diagnosis. Twenty-eight cases reported received HSCT, 6 out of these 28 patients died despite HSCT or during conditioning (**Table 1**).

Among the 15 cases of transplanted IPEX patients reported in detail (**Table 5**), half of them (8/15) received the transplant before 1 year of age, one of whom died. Among the other half, two patients who received the transplant at 9 and 13 years of age died of infections shortly after. More recently a 16-year-old patient underwent HSCT and a 1-year follow-up was reported. Despite the unfortunate outcome in some patients, the HSCT should be always recommended as the therapy of choice.

Both myeloablative and non-myeloablative conditioning regimens were used in order to limit complications associated with transplantation. The non-myeloablative regimens may enable reduction of both the post-transplant infectious complications and the toxicity of high dose chemotherapy. IPEX patients are very susceptible to the side effects of chemotherapy because of their poor clinical conditions. The use of a non-myeloablative conditioning can more easily result in a partial chimerism.

Both related and unrelated matched donors were used successfully. Only one patient received HSC from cord blood (Lucas et al., 2007) and three from mobilized peripheral blood (Zhan et al., 2008; Seidel et al., 2009; Burroughs et al., 2010), otherwise bone marrow was used as source of HSC (Baud et al., 2001; Wildin et al., 2002; Mazzolari et al., 2005; Rao et al., 2007; Dorsey et al., 2009).

The longest follow-up reported is approximately 8 years post-HSCT for three patients, including one patient transplanted at our Institute (unpublished observations: E. Mazzolari; M. Seidel; R. Bacchetta). Only one of these patients reached full-donor chimerism, however other cases with favorable outcome despite partial chimerism have been described. Therefore, complete donor engraftment in all hematopoietic lineages may not be necessary, but the preferential engraftment of donor Treg cells does indicate that at least the replacement of this cell subset is essential to cure the disease (Seidel et al., 2009). In light of this observation, the choice of drugs for GvHD prophylaxis should aim for the survival of donor Treg cells.

Since wild type Treg cells seem to be sufficient to control the disease, future cell/gene therapy approaches designed to selectively restore the repertoire of Treg cells represent a promising opportunity. Constitutive lenti-viral mediated overexpression of FOXP3 into $CD4^+$ T cells can convert Teff into Treg cells both in healthy subject (Allan et al., 2008) and in IPEX patients with different mutations (Passerini, in preparation). When a HLA compatible donor is not available, treatment with engineered T cells could be envisaged. Whether these cells would survive long enough to provide a stable life-long immune regulation without generalized immunosuppression remains to be clarified.

CONCLUSION

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome can be suspected on the basis of clinical and laboratory features, and the timely recognition of the disease leads to significant therapeutic benefits. A multicentre collaborative effort is desirable to implement studies in a wider cohort of patients, in order to achieve a complete knowledge of the disease, to better understand the factors that influence the outcome, and to identify new therapeutic targets. Functional impairment of Treg cells has been recognized as the primary defect at the basis of the immunodeficiency leading to autoimmunity in IPEX syndrome. However, there is evidence that *FOXP3* mutations can contribute to a complex immune-dysfunction, also involving Teff cells, and possibly other cell subsets. Immunological studies on IPEX syndrome have been instrumental in other PID to identify Treg dysfunctions, independent from FOXP3 mutations, as cause of autoimmunity and will most likely advance the knowledge and the therapeutic perspectives of other diseases with immune dysregulation of different origin.

ACKNOWLEDGMENTS

The authors thank the members of the Italian Study Group of IPEX (www.ipexconsortium.org). Our work is supported by the

Table 5 | Hematopoietic stem cell transplantation in IPEX patients.

	Baud et al. (2001)	Wildin et al. (2002)		Mazzolari et al. (2005)	Lucas et al. (2007)		Rao et al. (2007)			Zhan et al. (2008)	Seidel et al. (2009)	Dorsey et al. (2009)	Burroughs et al. (2010)		Kasow et al. (2011)
Age at onset	1 month	3 months	2 months	4 months	<1 year	na	2 months[§]	na	na	4 months	neonatal	neonatal	2 months	na	1.5 months
Age at BMT	6 months	13 years	9 years	9 months	6 years	7 years	1 years 5 months	4 years	6 months	5 months	11 months	7 months	9 months	16 years	7 months
Mutation	c.1113 T>G	c.1040G>A	c.748_750 delAAG	Promoter region	Exon 10	Intron 9	c.303_304 delTT	c.1271 G>A	c.1226 A>G	c.1139 C>T	c.3G>A	c.***878 A>G	c.210+2 delT	c.816+7 G>C	c.1150 G>A
Conditioning	ATG, Bu, Cx	Cx, TBI, ATG		Flu, Bu, Cx, ATG	Flu, Bu, ATG		ALM, Flu, Melph			ALM, Flu, Cx	ALM, Flu, Melph	ALM, Flu, Melph	Flu, TBI		ALM, Flu, Thio, Melph
$CD34^+$ source	BM	BM	BM	BM	CB	BM	BM	BM	BM	mPB	mPB	BM	mPB	BM	BM
Donor	MRD	MRD	MUD	MRD	MUD, 5/6°	MUD, 8/8°	MUD, 7/8°	MSD, 8/8°	MUD, 8/8°	MUD	MUD, 10/10°	MUD, 10/10°	MUD	MRD	MUD***, 10/10°
Chimerism (%)	100 → 30	100 → 50	100 → 70	70 (Treg 100)	98	na	na	na	na	100	90 Treg, 10 WBC	100	100	100	100 → 30
Remission	Yes*	Yes	Yes**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
GvHD	na	No	No	No	Gut 2°	Gut 2°	No	No	No	Skin-gut, 2–3°	Skin 2°	Skin 2°	Skin 2°	Gut 2°	No
Outcome	Exitus	Exitus	Exitus	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive

na, not available; ATG, anti-thymoglobulin; Bu, busulfan; Cx, cyclophosphamide; TBI, total body irradiation; Flu, fludarabine; ALM, alemtuzumab; Melph, melphalan; Thio, thiotepa; BM, bone marrow; CB, cord blood; mPB, mobilized peripheral blood; MRD, matched related donor; MUD, matched unrelated donor; GvHD, graft-versus-host disease.

*[§]The same case is reported also in Moudgil et al. (2007); °degree of HLA-match; *transient remission; **partial remission; ***T and B depleted.*

Telethon Foundation (Tele 10A4 to Rosa Bacchetta), the Italian Ministry of Health (Grant RF-2009-1485896 to Rosa Bacchetta), and the Seventh Framework project (FP7) of the European Community (Cell-PID to Rosa Bacchetta).The authors also thank Minso Kim, a medical student at New York University, for help in discussing and editing the present manuscript during her international stay at HSR-TIGET; Massimiliano Cecconi, Human Genetic Laboratory at Galliera Hospital, for helping in revision of the reported mutations, and Dr. Graziano Barera for useful discussion on neonatal enteropathies. Lastly, we are grateful to the patients and their families for their trust and participation in our studies.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 May 2012; accepted: 01 July 2012; published online: 31 July 2012.

Citation: Barzaghi F, Passerini L and Bacchetta R (2012) Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: a paradigm of immunodeficiency with autoimmunity. Front. Immun. ***3****:211. doi: 10.3389/fimmu.2012.00211*

This article was submitted to Frontiers in Primary Immunodeficiencies, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 18 July 2012
doi: 10.3389/fimmu.2012.00209

Autoimmunity in Wiskott–Aldrich syndrome: an unsolved enigma

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Wiskott–Aldrich Syndrome (WAS) is a severe X-linked Primary Immunodeficiency that affects 1–10 out of 1 million male individuals. WAS is caused by mutations in the WAS Protein (WASP) expressing gene that leads to the absent or reduced expression of the protein. WASP is a cytoplasmic protein that regulates the formation of actin filaments in hematopoietic cells. WASP deficiency causes many immune cell defects both in humans and in the WAS murine model, the *Was*$^{-/-}$ mouse. Both cellular and humoral immune defects in WAS patients contribute to the onset of severe clinical manifestations, in particular microthrombocytopenia, eczema, recurrent infections, and a high susceptibility to develop autoimmunity and malignancies. Autoimmune diseases affect from 22 to 72% of WAS patients and the most common manifestation is autoimmune hemolytic anemia, followed by vasculitis, arthritis, neutropenia, inflammatory bowel disease, and IgA nephropathy. Many groups have widely explored immune cell functionality in WAS partially explaining how cellular defects may lead to pathology. However, the mechanisms underlying the occurrence of autoimmune manifestations have not been clearly described yet. In the present review, we report the most recent progresses in the study of immune cell function in WAS that have started to unveil the mechanisms contributing to autoimmune complications in WAS patients.

Keywords: Wiskott–Aldrich syndrome, autoimmunity, primary immunodeficiency, T lymphocytes, B lymphocytes

WISKOTT–ALDRICH SYNDROME: CELLULAR DEFECTS AND CLINICAL MANIFESTATIONS

Wiskott–Aldrich Syndrome (WAS) is a rare X-linked Primary Immunodeficiency (PID) that affects 1–10 out of a million male individuals (Ochs and Thrasher, 2006), whose life expectancy is about 15 years in severe cases (Imai et al., 2004). Affected patients demonstrate both cellular and humoral immunodeficiency, high susceptibility to infections, eczema, microthrombocytopenia, and increased risk of autoimmune disorders and lymphomas (Bosticardo et al., 2009). WAS is caused by defective expression of WAS Protein (WASP), a key regulator of cytoskeletal organization in hematopoietic cells (**Figure 1**). The *WAS* gene is located on the X chromosome and encodes a 502 amino acid protein (Derry et al., 1994), which is constitutively expressed in the cytoplasm of hematopoietic cells (Kim et al., 2000). WASP is present in an auto-inhibited conformation and its activation is mainly induced by the binding with GTPase Cell division Cycle 42 (CDC42; Abdul-Manan et al., 1999). Other factors, such as the Non-Catalytic region of tyrosine Kinase adaptor protein (NCK; Tomasevic et al., 2007), and the phosphorylation of WASP tyrosine residue 291 (Y291) can activate WASP independently of CDC42 (Cory et al., 2002; Badour et al., 2004). The binding of Phosphatidylinositol-4,5-bisphosphate (PIP_2) is also an important regulator of WASP activation by inducing a stable acting form (Imai et al., 1999). WASP, in the active form, binds the Actin-Related Protein (ARP)2/3 complex, which gives rise to nucleation of actin filaments at the side of pre-existing filaments, thus creating a branching network of actin at the plasma membrane (Symons et al., 1996; Machesky and Insall, 1998; Miki et al., 1998; Machesky and Gould, 1999; Blanchoin et al., 2000; Pantaloni et al., 2000). The activity of the ARP2/3 complex was shown to contribute to a variety of cellular functions, including change of cell shape, motility, endocytosis, and phagocytosis (Welch and Mullins, 2002).

The severity of disease, measured on the basis of the classification proposed by Zhu et al. (1997) and subsequently modified (Ochs and Thrasher, 2006; Ochs et al., 2009), is schematically reported in **Table 1**.

A score from one to two identifies patients affected from a milder form of the disease, named X-Linked Thrombocytopenia (XLT; Villa et al., 1995), and characterized by reduced expression of full-length mutated protein and microthrombocytopenia. Localized eczema and occasional respiratory infections, in addition to microthrombocytopenia, identify score 2 of the disease. Patients who develop microthrombocytopenia, associated with persistent but therapy-responsive eczema or infections receive a score of 3, whereas a score of 4 is given if eczema or infections do not respond to treatments. Finally, score 5 is assigned to patients developing autoimmunity or tumors.

Wiskott–Aldrich Syndrome gene mutations are scattered throughout the entire length of the *WAS* gene, although some hot spots have been identified (Ochs and Thrasher, 2006). Mutations that abolish WASP expression are mainly associated with a

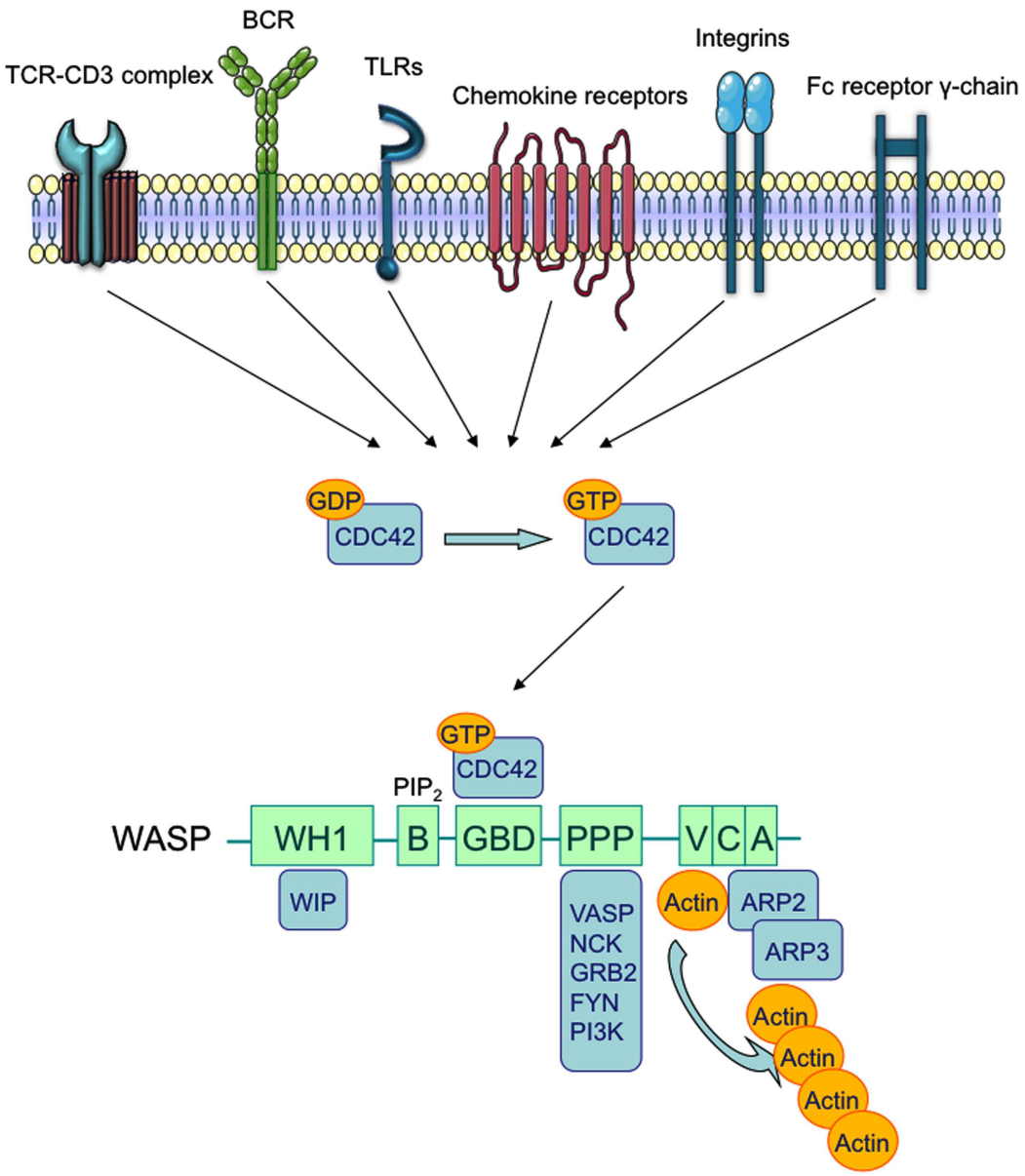

FIGURE 1 | Wiskott–Aldrich syndrome structure and interacting proteins. TCR, BCR, chemokine receptors, TLRs, integrins, and the Fc receptor γ-chain can promote the release of GDP from Rho family GTPases, allowing GTP to bind. In immune cells, the major Rho GTPase is the Cell Division Cycle 42 (CDC42). The WASP-Homology 1 (WH1) domain mediates the binding to WASP-Interacting Protein (WIP; Ramesh et al., 1997). The Phosphatidylinositol-4,5-bisphosphate (PIP_2) links to the Basic (B) domain and stabilizes WASP active form. The binding of the GTPase-Binding Domain (GBD) with CDC42 induces WASP activation (Kolluri et al., 1996; Symons et al., 1996; Miki et al., 1998). The proline-rich region (PPP) provides binding sites for the Vasodilator-Stimulated Phosphoprotein (VASP), and also for SRC family tyrosine kinases and SRC Homology 3 (SH3) domain-containing proteins such as the adaptor proteins GRB2, FYN, PI3K, and NCK. The Verprolin-homology (V) domain binds to actin monomers, and the Cofilin-homology (C) and Acidic (A) domains bind to the Actin-Related Protein (ARP)2/3 complex. The V/C/A region functions as the platform to initiate actin polymerization (Park et al., 2010).

severe clinical phenotype (full blown WAS) and a life expectancy below 20 years of age (Jin et al., 2004). On the contrary, missense mutations, which result in residual expression of a full-length point-mutated WASP, are often associated with XLT (Villa et al., 1995; Notarangelo et al., 2002; Albert et al., 2010), corresponding to a disease score of 0.5–2 and a longer life expectancy (Imai et al., 2004). All patients harboring mutations in the *WAS* gene are micro-thrombocytopenic, although intermittent X-Linked Thrombocytopenia (iXLT) is observed in some patients with substantial protein expression (Notarangelo et al., 2002). Importantly, up to 11% of patients can present somatic mosaicism due to spontaneous *in vivo* reversion of the original mutation or second-site compensatory mutations that restore production of the *WAS* gene product (Stewart et al., 2007). The revertant mutation can occur

Table 1 | WAS scoring system according to Zhu et al. (1997), with subsequent refinements (Ochs and Thrasher, 2006; Ochs et al., 2009).

Clinical scores	iXLT	XLT		WAS		
	<1	1	2	3	4	5
Thrombocytopenia	−/+	+	+	+	+	+
Small platelets	+	+	+	+	+	+
Eczema	−	−	(+)	+	++	(+)/+/++
Immunodeficiency	−	−/(+)	(+)	+	+	(+)/+
Infections	−	−	(+)	+	+/++	(+)/+/++
Autoimmunity or malignancy	−	−	−	−	−	+

Scoring system: −, absent; (+), mild; +, present; ++, present and severe. iXLT, intermittent X-linked thrombocytopenia; WAS, Wiskott–Aldrich syndrome; XLT, X-linked thrombocytopenia.

at various stages of hematopoietic differentiation thus conferring high selective advantage to revertant cells over mutated cell populations not expressing WASP. Although many reports describe the occurrence of this phenomenon, it is still not clear whether the presence of somatic mosaicism might correlate with a better clinical course of the disease (Davis and Candotti, 2009; Trifari et al., 2010).

Absence or residual WASP expression causes functional defects in all immune cells (**Figure 2**).

The formation of the Immunological Synapse (IS) in T cells and T Cell Receptor (TCR)-dependent activation (Dupre et al., 2002; Trifari et al., 2006; Nikolov et al., 2010), the cytotoxic activity of $CD8^+$ T cells and Natural Killer (NK) cells (Orange et al., 2002; de Meester et al., 2010) and the suppressor activity of Naturally occurring Regulatory T (nTreg) cells (Adriani et al., 2007, 2011; Humblet-Baron et al., 2007; Maillard et al., 2007; Marangoni et al., 2007) are all impaired in WASP-deficient cells. Motility, adhesion and migration of B cells are also defective (Westerberg et al., 2005; Meyer-Bahlburg et al., 2008). Additionally, the lack of WASP affects podosome formation (Burns et al., 2001; Calle et al., 2004), motility (Binks et al., 1998; de Noronha et al., 2005) and T cell priming in Dendritic Cells (DCs; Pulecio et al., 2008; Bouma et al., 2011), as well as podosome and phagocytic cup formation in macrophages (Linder et al., 1999; Tsuboi and Meerloo, 2007). Invariant Natural Killer T (iNKT) cell functionality (Astrakhan et al., 2009; Locci et al., 2009), adhesion, and migration of neutrophils (Zhang et al., 2006) are also altered in the absence of WASP. Moreover, WASP is also involved in signal transduction (**Figure 3**). In particular, TCR-dependent nuclear recruitment of Nuclear Factor of Activated T cells (NFAT)-1 in $CD4^+$ T cells and both NFAT-1 and NFAT-2 in $CD8^+$ T cells are reduced in WAS patients and correlate with defective Th1 cytokine production (Cianferoni et al., 2005; Trifari et al., 2006). Additionally, WASP is involved in B Cell Receptor (BCR) signaling by binding to the Src homology three domains of several tyrosine kinases, such as the Bruton's Tyrosine Kinase (BTK; Cory et al., 1996; Sharma et al., 2009).

The most common finding in WAS patients is microthrombocytopenia which causes frequent hemorrhages in more than 80% of patients (Ochs, 2002; Imai et al., 2004) and severe bleeding episodes that lead to death in 4–10% of patients (Sullivan et al., 1994; Imai et al., 2004). The mechanism underlying thrombocytopenia is not completely understood. One possible explanation could be an abnormal platelet clearance induced by an increased exposure of phosphatidylserine on the outer plasma membrane of WASP-deficient platelets (Shcherbina et al., 2009). Another mechanism of platelet reduction that needs to be investigated more in detail, is the elimination mediated by autoimmune reaction. In fact, the presence of platelet-associated antibodies in $Was^{-/-}$ mice (Marathe et al., 2009) and in some patients has been reported (Corash et al., 1985; Semple et al., 1997). The second most common manifestation in WAS patients is the eczema. It is observed in 80% of patients and its severity inversely correlates with the expression of WASP. Indeed, it has been shown that WAS patients with residual WASP expression develop moderate or transient form of the disease, whereas most of WASP-negative patients develop severe, treatment-resistant eczema (Imai et al., 2004). High IgE levels (more than 1000 IU/mL) were observed in 62% of WASP-negative patients and in 25% of WASP-positive. Although higher IgE levels may represent a possible cause of eczema, the correlation between increased IgE levels and eczema has not yet been demonstrated. WAS patients are highly susceptible to infections by bacteria, viruses, and fungi (Imai et al., 2004). Of note, WASP-negative patients are more frequently affected by bacterial infections (otitis media, skin abscess, pneumonia, enterocolitis, meningitis, sepsis, urinary tract infection, and others), viral infections (*Herpes simplex* and *Cytomegalovirus*) and fungal infections (*Candida* spp., *Aspergillus* spp., and *Pneumocystis carinii*) as compared to WASP-positive patients (Imai et al., 2004). Patients with clinically most severe WAS develop malignancies and/or autoimmune manifestations. Malignancies can affect adolescent and young adult WAS patients more than infants (Sullivan et al., 1994; Imai et al., 2004). Epstein–Barr virus (EBV)-positive B cell lymphoma is most frequently reported, but also myelodysplasia can be observed in some patients (Imai et al., 2004). Autoimmune complications are frequently observed in WAS, affecting 22–72% of patients (Dupuis-Girod et al., 2003; Imai et al., 2004). WAS patients with autoimmune diseases constitute a high-risk group with poor prognosis. Moreover, autoimmunity is associated with a higher risk of a later development of tumors and an increased risk of mortality (Sullivan et al., 1994). A better understanding of the mechanisms underlying autoimmunity in WAS would be crucial for the development of more effective therapies for the management of these manifestations in WAS and could also provide new insights in the pathogenesis of autoimmunity in PIDs.

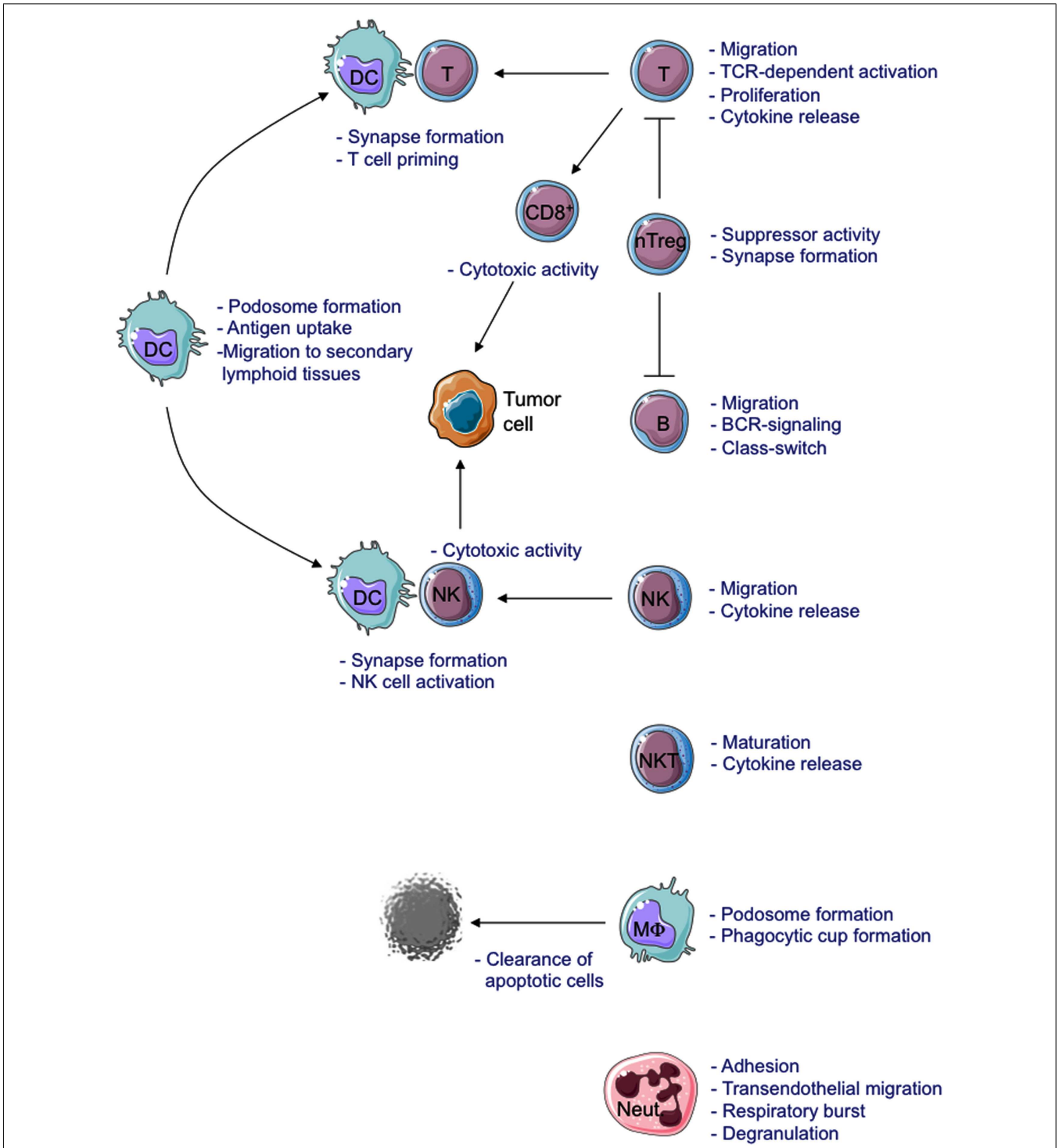

FIGURE 2 | Schematic view of cellular defects described in WASP-deficient cells. MΦ, Macrophage; Neut., Neutrophil.

AUTOIMMUNE MANIFESTATIONS IN WAS PATIENTS AND CURRENT TREATMENTS

The most common autoimmune manifestation in WAS is hemolytic anemia (36%), followed by vasculitis (including cerebral vasculitis; 29%), arthritis (29%), neutropenia (25%), inflammatory bowel disease (9%), and IgA nephropathy (3%). Henoch–Schönlein-like purpura, dermatomyositis, recurrent angioedema, and uveitis have also been reported in some patients (Dupuis-Girod et al., 2003; Imai et al., 2004). Moreover, multiple autoimmune manifestations can be observed. In most cases, and

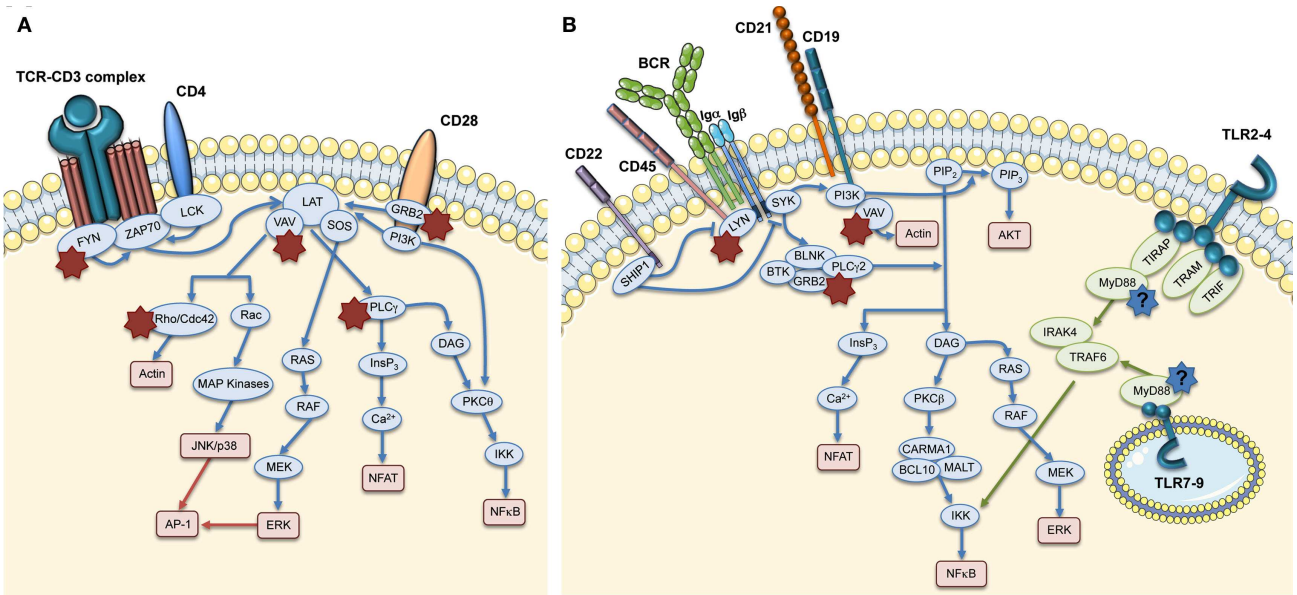

FIGURE 3 | Role of WASP in TCR and BCR signaling pathways. The main signaling molecules (blue and green ovals) acting downstream of TCR **(A)**, BCR, and TLRs **(B)** are depicted. The red boxes indicate the main pathways induced downstream of these receptors. The role of WASP in these pathways is indicated by stars close to interacting molecules: red stars show the interactions that are demonstrated, whereas the blue stars with question marks show hypothetical involvement of WASP in TLR signaling pathways.

in all cases of hemolytic anemia, the onset of autoimmune complications occurs early in life (0–5 years; Dupuis-Girod et al., 2003). Interestingly, it has been recently found that *Was*$^{-/-}$ mice develop proliferative glomerulonephritis with increased IgA in the serum and IgA production by splenic B cells (Nikolov et al., 2010; Shimizu et al., 2012). Moreover old *Was*$^{-/-}$ mice showed aberrant glycosylation of IgA (Shimizu et al., 2012), feature that has been associated to the development of nephropathy-like glomerular lesions with IgA deposition (Nishie et al., 2007). Although these studies have been performed on WAS mouse model, they clearly suggest a possible mechanism for the pathogenesis of glomerulonephritis in WAS patients.

Clinical management of WAS patients is a significant challenge since, with the exception of Bone Marrow Transplantation (BMT), most available therapies are not curative. Intravenous immunoglobulins (IVIG) and antibiotic prophylaxis are often used to reduce the risk of infections in WAS patients, but it is not clear whether these treatments effectively reduce the incidence of life-threatening infections (Conley et al., 2003). Splenectomy significantly increases and often normalizes the platelet counts (Corash et al., 1985; Mullen et al., 1993). However, it does not fully overcome the risk of bleeding and further predisposes to sepsis, obliging the patients to life-long antibiotic prophylaxis (Mullen et al., 1993). Relapse of thrombocytopenia has been described in a fraction of splenectomized WAS patients (Corash et al., 1985; Dupuis-Girod et al., 2003). Of note, in some cases, thrombocytopenia was found to be autoantibody-mediated and also associated with hemolytic anemia or cerebral vasculitis (Dupuis-Girod et al., 2003). Therefore, splenectomy is indicated only in severe cases, for which there is no prospect for other curative interventions. Treatment with human recombinant Interleukin 2 (hrIL-2) appeared to be effective in reducing herpes virus infections and improving dermatitis in a WAS patient (Azuma et al., 1993). Administration of hrIL-2 ameliorated proliferation of cultured T cells from one patient (Azuma et al., 2000) and restored cytotoxicity and actin accumulation at the IS in NK cells from another treated patient (Orange et al., 2011). Since WAS T cells are less efficient in producing IL-2, NK cells do not receive sufficient IL-2, thus resulting in reduced NK activation and failure to respond effectively to infections. A clinical trial with hrIL-2 is currently ongoing in WAS (ClinicalTrials.gov identifier NCT00774358). The treatment of choice for autoimmune manifestations in WAS patients consists of steroids, alone, or in association with cyclosporine (Dupuis-Girod et al., 2003). Steroids are the first-line treatment for all patients with hemolytic anemia and efficiently induce remission in 10% of cases, are partially effective in 60% of cases, while are ineffective in 30% of cases. Cyclophosphamide and azathioprine are also used in some cases and are effective in a small percentage of cases. Patients with severe autoimmune thrombocytopenia after splenectomy are usually treated with IVIG, high-dose steroids, azathioprine, and cyclophosphamide. Other autoimmune or inflammatory complications are generally treated with steroids, in association with cyclosporine, and are effective in the majority of skin vasculitis, arthritis, bowel inflammatory disease and renal disease cases (Dupuis-Girod et al., 2003). Anti-CD20 monoclonal antibody therapy has been also performed for the treatment of autoimmune hemolytic anemia in some patients. This treatment results effective in correcting the anemia, but it may need repeated courses due to relapse of the disease (Ship et al., 2002; Kim et al., 2007).

Currently, the only resolutive therapeutic option for WAS patients is BMT. When a Related HLA-Identical Donor (RID) is

available, BMT leads to 73–100% survival (Mullen et al., 1993; Ozsahin et al., 1996, 2008; Filipovich et al., 2001; Antoine et al., 2003; Kobayashi et al., 2006; Pai et al., 2006; Moratto et al., 2011). On the other hand, transplantation using the bone marrow of a Mismatched Related Donor (MMRD) results in a poor survival ranging from 29 to 52% (Mullen et al., 1993; Filipovich et al., 2001; Kobayashi et al., 2006; Ozsahin et al., 2008). In addition, this type of transplant is associated with an elevated risk of developing life-threatening EBV^+ lymphoproliferative syndrome, infections, autoimmunity, and graft-versus-host disease (GVHD; Filipovich et al., 2001), therefore it is not recommended except in case of emergency. When a suitable related donor is missing, transplantation using the bone marrow or cord blood from a Matched Unrelated Donor (MUD) is a valid therapeutic option, leading to 71–81% survival (Filipovich et al., 2001; Kobayashi et al., 2006; Pai et al., 2006). Two recent retrospective studies have analyzed long-term outcome and donor cell engraftment in WAS patients who have been treated by Hematopoietic Stem Cell Transplantation (HSCT; Ozsahin et al., 2008; Moratto et al., 2011). They observed that 20% of patients developed autoimmune manifestations after HSCT independently of chronic GVHD (Ozsahin et al., 2008) and some patients had more than one manifestation. Autoimmune manifestations appeared at a median of 1.5 years after HSCT (range: 4 months to 10 years). The median duration of autoimmunity was 4 years (range: 1–20 years). Autoimmune manifestations were more frequent in recipients of MUD (28%) and MMRD (26%) than RID HSCT (11%). Ozsahin and colleagues investigated whether patients developing autoimmunity after HSCT had autoimmune manifestations also before treatment. Overall, 17 patients had autoimmune manifestations before transplantation that persisted thereafter in seven of them. Conversely, autoimmunity occurred *de novo* in 11–23% of transplanted patients. A very interesting observation in both retrospective studies was the strong correlation between autoimmunity occurrence and the chimerism pattern. Overall, incomplete reconstitution of lymphocyte counts and incidence of autoimmunity were higher in patients with a lower degree of chimerism in both lymphoid and myeloid compartments as compared to patients with full chimerism (Ozsahin et al., 2008; Moratto et al., 2011).

A very promising alternative to HSCT, when a matched donor is missing, is the infusion of gene corrected autologous Hematopoietic Stem Cells (HSCs). Two different Gene Therapy (GT) clinical trials have been approved: a Retroviral Vector (RV)-mediated gene transfer (Boztug et al., 2010) and a Lentiviral Vector (LV)-mediated GT approach, developed by our and other groups (Dupre et al., 2006; Galy et al., 2008). In the RV-mediated clinical trial, sustained expression of WASP in HSCs, lymphoid and myeloid cells, and platelets was shown in two treated patients 3 years after GT (Boztug et al., 2010). T and B cells, NK cells, and monocytes were also functionally corrected resulting in improved clinical conditions. Signs and symptoms of autoimmunity disappeared in both patients within the first year after GT. In one of the two reported patients, severe autoimmune hemolytic anemia, autoimmune thrombocytopenia, and autoimmune neutropenia disappeared; whereas severe eczema resolved in the second patient. However, in this trial, leukemia occurred in one out of ten GT patients, probably due to insertional mutagenesis caused by RV integration (Press Release, Hannover Medical School, http://www.asgct.org/UserFiles/file/Genetherapy_WAS_final_english.pdf). This adverse event gives rise to some concerns on the safety of RV-mediated GT for WAS. A multicenter clinical trial using a third generation LV carrying *WAS* gene driven by the endogenous promoter is on going in Milan, Paris, and London. Preclinical data in the murine model indicate that the LV-mediated GT approach is effective in restoring immune cell functionality (Blundell et al., 2008; Marangoni et al., 2009; Bosticardo et al., 2011; Catucci et al., 2011). GT treated $Was^{-/-}$ mice did not show any adverse events or tumors even in long-term follow up studies (Marangoni et al., 2009). Finally, we and others demonstrated the efficacy of LV-mediated GT in $CD34^+$ cells obtained from WAS patients (Charrier et al., 2007; Scaramuzza et al., 2012). Nevertheless, data from the clinical study are needed to provide definitive evidence of the efficacy and safety of this novel therapeutic approach.

REGULATION OF T CELL TOLERANCE IN WAS

T cells are significantly reduced in peripheral blood of WAS patients and show a defective proliferation in response to TCR stimulation by CD3-specific antibody, although this defect is present only at low doses of agonistic antibody (Molina et al., 1992, 1993). TCR-dependent activation in WASP-deficient T cells results in a reduced IL-2 production (Molina et al., 1993), that is associated with delayed NFAT-1 nuclear translocation and defective T-bet induction (Cianferoni et al., 2005; Trifari et al., 2006; Taylor et al., 2010). T cell activation is regulated by the formation of the IS, a polarized cluster of TCR, costimulatory molecules, signaling molecules, and integrins at the T cell:antigen presenting cell (APC) interface. To promote their lateral movement on the plasma membrane, the molecules being recruited to the IS are associated with specific cholesterol-enriched membrane microdomains, called lipid rafts. In the absence of WASP, IS can be formed only after strong TCR stimulation (Cannon and Burkhardt, 2004). In particular, WASP-deficient T cells fail to upregulate GM1 on the cell surface, cluster GM1 in the lipid rafts during IS formation (Dupre et al., 2002) and maintain IS stability after migration (Sims et al., 2007).

It is commonly assumed that autoimmunity is a consequence of the breakdown of central or peripheral tolerance to self-antigens. nTreg cells are fundamental to maintain tolerance to self-antigens and suppress excessive immune responses. nTreg cell development and function depend on TCR signaling, together with CD28 recruitment, FOXP3 expression, and presence of IL-2 (Sakaguchi et al., 2008). Several groups, including ours, have described the defects of nTreg cells in WAS patients and $Was^{-/-}$ mice in localizing and suppressing T effector cell response (Adriani et al., 2007; Maillard et al., 2007; Marangoni et al., 2007), although their number in blood of WAS patients is comparable with healthy donors (Marangoni et al., 2007). It is not clear whether a defective thymic development of $Was^{-/-}$ nTreg cells could account for their impaired *in vivo* suppressive function, since one group has shown reduced nTreg cell percentage in the thymus (Maillard et al., 2007), while three other groups observed normal frequency while showed a reduced function *in vivo* (Adriani et al., 2007; Humblet-Baron et al., 2007; Marangoni et al., 2007), but all showed a reduced *in vivo* suppression. $Was^{-/-}$ nTreg cell failure to control

aberrant T cell activation has been also demonstrated *in vivo* in a mouse model of autoimmunity (Humblet-Baron et al., 2007). Moreover, selective advantage of WASP-expressing nTreg cells was shown in a WAS patient with revertant mutation, demonstrating that WASP has a role in nTreg cell fitness (Humblet-Baron et al., 2007). Although the requirement of WASP for nTreg cell functionality has been demonstrated, the role of WASP in these cells is still unclear. Indeed, differently from effector T cells, WASP is not recruited to the IS (Marangoni et al., 2007), thus suggesting a possible role of WASP in TCR signaling of nTreg cells. Moreover, WASP-deficient nTreg cells are also defective in suppressing B cell activation. In fact, it has been shown in *in vitro* studies that nTreg cells from *Was*$^{-/-}$ mice are less efficient in turning off B cell proliferation and this defect is associated with a reduced killing of B cells and significantly decreased secretion of granzyme B by nTreg cells (Adriani et al., 2011). Susceptibility of WAS patients to develop autoimmune diseases can be at least in part explained by nTreg cell dysfunction.

Recent findings have demonstrated that also T effector cells are implicated in tolerance breakdown in WAS. Indeed, in response to restimulation through the TCR, activated T cells can undergo apoptosis, and this event is called restimulation-induced cell death (RICD; Lenardo, 1991; Siegel et al., 2000). RICD process contributes to the maintenance of peripheral immune tolerance by eliminating T cells responding to prolonged presence of antigens, such as self-antigens and persistent pathogen antigens (Critchfield et al., 1994; Ettinger et al., 1995; Weant et al., 2008). In CD4^{+} T cells, RICD is induced by the Tumor Necrosis Factor (TNF) family member Fas ligand (FasL) that is released and binds its receptor Fas in an autocrine fashion (Critchfield et al., 1994; Ettinger et al., 1995; Siegel et al., 2000; Green et al., 2003; Weant et al., 2008). Nikolov and colleagues have shown that WASP is required for T cell apoptosis by RICD. In the absence of WASP, the release of FasL by CD4^{+} T cells is reduced and this is associated to a decreased TCR-mediated apoptosis (Nikolov et al., 2010). Together with nTreg cell defects, these recent findings highlight the role played by effector T cells in the maintenance of T cell tolerance in WAS.

REGULATION OF B CELL TOLERANCE IN WAS

In the last years, many studies have assessed the role of B cells in driving autoimmune diseases such as Rheumatoid Arthritis (RA), Multiple Sclerosis (MS), and Systemic Lupus Erythematosus (SLE; Townsend et al., 2010). These data revealed the complex role of B cells that work independently or synergistically with other components of the innate and adaptive immune system to drive autoimmune pathogenesis.

For many years, the functionality of B cells in WAS patients was poorly investigated. The presence of a skewed distribution of serum Ig isotypes (reduced IgM, normal IgG, and elevated IgE and IgA levels) and a reduced or absent antibody production to polysaccharides and other T cell-independent antigens (Golding et al., 1984; Ochs and Thrasher, 2006) represent the first evidences of a defective B cell effector function in WAS patients. This prompted many researchers to investigate more in detail the B cell compartment in WAS, mainly taking advantage of the murine model of the disease (*Was*$^{-/-}$ mice). In the last decade, it has been clearly defined that the lack of WASP causes defects in the cytoskeletal functions of B cells, including adhesion, migration, and homing (Westerberg et al., 2005, 2008; Meyer-Bahlburg et al., 2008). These defects may compromise the capacity of B cells to be properly activated and reach the site of infection contributing to the inability of the immunodeficient host to completely eradicate infectious agents. In this respect, it has been accepted, in particular for PIDs, that chronic immune response due to an incomplete pathogen clearance may favor breakdown of peripheral tolerance. Of note, the complement receptors CD21 (CR2) and CD35 (CR1) are expressed at lower levels on B cells of patients with WAS (Park et al., 2005) contributing to a suboptimal B cell capacity to capture and present opsonized antigens. Additionally, given the critical role of CD21 and CD35 in the negative selection of self-reactive B cells (Prodeus et al., 1998), the altered expression or function of these receptors may affect the maintenance of B cell tolerance in Immune Complex (IC)-mediated autoimmune diseases such as SLE and RA (Erdei et al., 2009).

The fate of self-reactive B cells within the bone marrow and peripheral lymphoid compartment is largely determined by the strength of signal mediated by BCR in response to antigen cross-linking (Nemazee and Burki, 1989; Erikson et al., 1991; Goodnow, 1996). To this regard, reports of a defective BCR activation are controversial. Activation of WASP-deficient B cells was found to be defective after BCR engagement in terms of calcium mobilization in primary B cells isolated from WAS patients and also in WASP-deficient EBV-transformed B cell lines (Simon et al., 1992). However, this defect was not confirmed by Henriquez et al. (1994). More recently, studies performed on *Was*$^{-/-}$ mice showed a normal proliferative response of B cells after stimulation with anti-IgM, LPS, or anti-CD40 (Snapper et al., 1998; Zhang et al., 1999) and a normal or increased class switch (Westerberg et al., 2005). However, the presence of circulating autoantibodies in WAS patients (Dupuis-Girod et al., 2003; Schurman and Candotti, 2003) and in *Was*$^{-/-}$ mice (Humblet-Baron et al., 2007; Nikolov et al., 2010; Becker-Herman et al., 2011; Bosticardo et al., 2011) represents the first evidence of a perturbed B cell tolerance. Very recently, two studies have shown the contribution of B cell intrinsic defects to the pathogenesis of autoimmunity in two different murine models. Indeed, Becker-Herman et al. (2011) observed that female *Was*$^{+/-}$ mice generate anti-nuclear antibodies at rates and titers equivalent to *Was*$^{-/-}$ mice even though heterozygous animals have a normal nTreg cell compartment. Based on this evidence, they demonstrated in mixed BM chimeras, in which only B cells lacked WASP expression, that the selective defect in B cells is sufficient for the generation of autoantibodies. Additionally, they suggested that BCR/Toll-Like Receptor (TLR) co-engagement in *Was*$^{-/-}$ B cells from chimeras could mediate tolerance breakdown, since the loss of Myeloid Differentiation primary response gene 88 (MyD88) signaling abolished the production of anti-dsDNA antibodies, germinal center formation, and development of systemic autoimmune disease (Becker-Herman et al., 2011). More recently, by conditional *WAS* gene deletion in B cells (B/WcKO mice), Recher et al. (2012) observed that WASP deficiency limited to B cells is sufficient to promote autoantibody production and kidney tissue damage in B/WcKO mice.

Overall, these findings highlight the contribution of B cells to the pathogenesis of autoimmunity in WAS and suggest that the B

cell autonomous defect could represent a sufficient factor to break tolerance in WAS. However, in addition to the supposed role for TLR signaling in the autoreactivity of *Was*$^{-/-}$ B cells, other mechanisms are potentially involved and need to be further investigated both in mice and humans.

FUTURE DIRECTIONS

WAS is characterized by a very complex spectrum of cellular defects, many of which can predispose patients to the development of autoimmunity (**Figure 4**).

As described above, defective control of the strength of immune response by nTreg cells, the presence of autoantibodies and potentially autoreactive B cells have been demonstrated in WAS (Boscardo et al., 2009). However several mechanisms shown to be involved in the pathogenesis of autoimmune diseases still need to be investigated in WAS. iNKT cells have been shown to prevent autoimmune disease in a mouse model of experimental Autoimmune Encephalomyelitis (EAE; Miyamoto et al., 2001; Singh et al., 2001) and uveitis (Oh et al., 2011). Although the mechanisms have not been fully understood, it has been shown that iNKT activation reduces autoimmune symptoms by limiting the development of Th17 cells in a cell contact- and cytokine-dependent manner (Mars et al., 2009). Moreover, in mice, iNKT cells suppress anti-DNA antibody production and reduce autoreactive B cells (Yang et al., 2011), whereas iNKT reduction leads to increased autoreactive B cell activation (Wermeling et al., 2010). Since iNKT cells are reduced in WAS patients and functionally defective in *Was*$^{-/-}$ mice (Astrakhan et al., 2009; Locci et al., 2009) it can be hypothesized that such impairment may also contribute to autoimmunity.

A mechanism contributing to the tolerance breakdown in PIDs is related to the inability of innate immune cells, in particular DCs, to properly activate adaptive immune response (Arkwright et al., 2002). Since DCs have a role in the induction of nTreg cells (Manicassamy and Pulendran, 2011) and DCs lacking WASP are defective in T cell priming (Bouma et al., 2007; Pulecio et al., 2008), it is

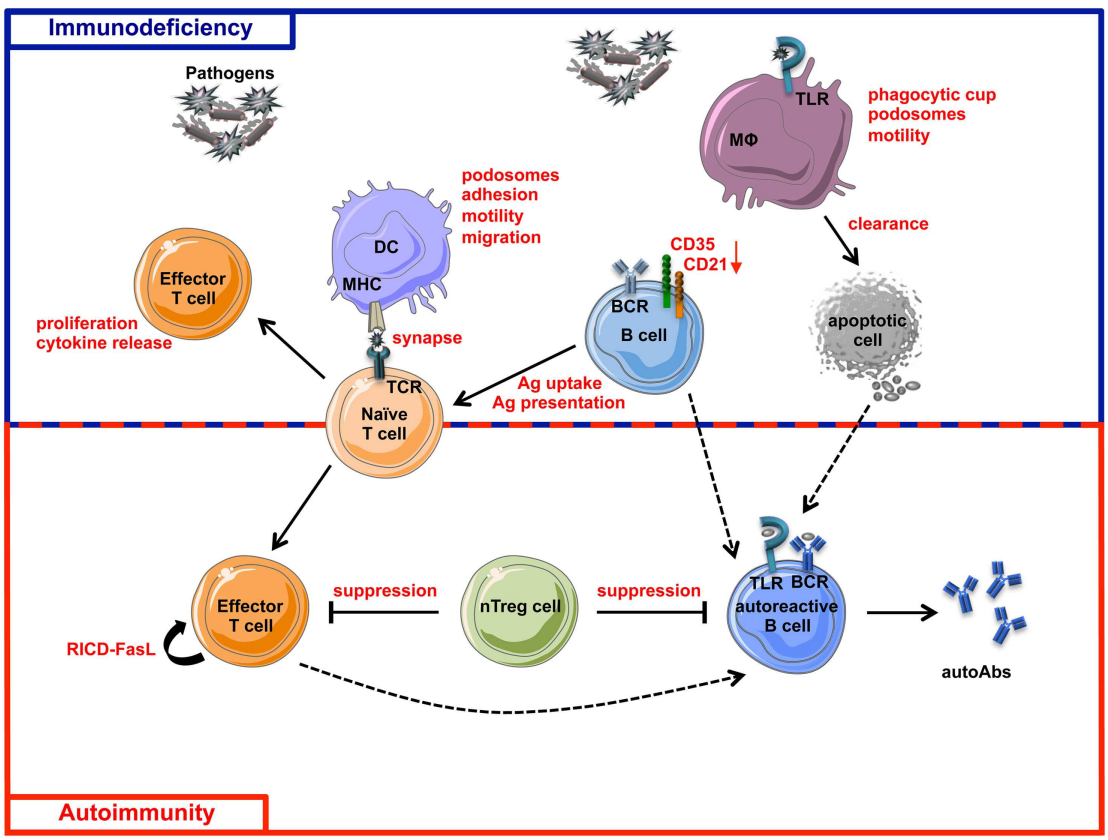

FIGURE 4 | Schematic view of immunodeficiency and autoimmunity in WAS. The impairment of both innate and adaptive immune systems is responsible of immunodeficiency (blue box) and autoimmunity (red box) in WAS. Immune cell defects described in WASP-deficient cells are shown in red. The incomplete pathogen clearance is sustained by cytoskeleton and functional defects of macrophages, DCs, T cells, B cells, and their defective interactions. The reduced expression of CD21 and CD35, two complement receptors involved in antigen uptake and presentation and also in negative selection of self-reactive B cells, places B cells at the interface between immunodeficiency and autoimmunity. Defective suppression of WASP-deficient nTreg cells toward both T and B cells contributes to the tolerance breakdown in WAS. Defect in RICD process, resulting in defective effector T cell apoptotic death after TCR restimulation, concurs in the persistence of T cell response to pathogens or self-antigens. Additionally, intrinsic B cell defects contribute to autoimmunity in WAS, probably via a TLR-mediated mechanism. Dashed lines represent hypothetical mechanisms involved in WAS-related autoimmunity. MHC, Major Histocompatibility Complex; RICD, Restimulation-Induced Cell Death; autoAbs, autoantibodies; Ag, Antigen.

possible to hypothesize that a defect in nTreg cell induction by DCs might occur in the absence of WASP. Immunodysregulation can be also sustained by overload of pathogen antigens or apoptotic material due to defective clearance by innate immune cells. Antigen overload in fact results in a prolonged immune response, which promotes expansion of Th17 cell subset, playing a central role in many autoimmune diseases, such as MS, RA, and Crohn's disease (Langrish et al., 2005; Fouser et al., 2008; Isaksson et al., 2009; Sharma et al., 2009). Furthermore, reduced clearance of apoptotic material has been associated to the accumulation of autoantibodies in SLE (Gaipl et al., 2005; Fransen et al., 2012). WASP-deficient DCs are impaired in antigen uptake and migration to secondary lymphoid tissues (Westerberg et al., 2003; de Noronha et al., 2005) suggesting an inefficient pathogen clearance, process that needs to be investigated in *in vivo* models of infection. Moreover, WASP-deficient macrophages are less efficient in uptaking apoptotic cells both *in vitro* and *in vivo* (Leverrier et al., 2001). All together, these findings suggest that dysregulation of Th17 cell activation might contribute to autoimmunity induction in WAS patients, although no evidence has been provided so far to sustain such hypothesis.

Recent studies have demonstrated the key role played by a specific subset of DCs, namely plasmacytoid DCs (pDCs), in the pathogenesis of systemic autoimmune diseases. In particular, IFN-α produced by pDCs upon recognition of foreign nucleic acids via TLR7 and TLR9 contributes to tolerance breakdown in several autoimmune diseases, such as SLE, SS, and psoriasis (Ronnblom, 2011). In these clinical settings, self-nucleic acid-containing ICs trigger TLR7 or TLR9 leading to an uncontrolled pDCs activation. In PIDs, an increased susceptibility to viral infection, in combination with a defective clearance of pathogens, could be the triggering factor of the over-activation of type I IFN pathway. Moreover, cell death induced by viral infection leads to the release and accumulation of self-antigens in the extracellular matrix. Since PID patients, including WAS patients, are highly susceptible to infections and fail to completely eradicate the pathogens, high levels of ICs and activation of the type I IFN system can be expected. Furthermore, increasing evidences highlight the role played by neutrophils in SLE in the induction of type I IFN production. Mature neutrophils are primed *in vivo* by type I IFN and die upon exposure to anti-ribonucleoprotein antibodies, releasing neutrophil extracellular traps (NETs) which in turn activate pDCs to produce high levels of type I IFN (Garcia-Romo et al., 2011). Overall, these studies have demonstrated an important role of neutrophils and pDCs in promoting autoimmune diseases and it can be envisaged that these mechanisms may act in the complexity of WAS autoimmunity.

Triggering of autoreactive B cells by self-nucleic acid-containing ICs can be another possible mechanism underlying the production of autoantibodies in WAS. In fact, it is known that self-nucleic acid-containing ICs can activate B cells through synergistic engagement of BCR and TLR7 or TLR9 (Leadbetter et al., 2002; Lau et al., 2005; Chaturvedi et al., 2008), and the loss of MyD88 signaling in $Was^{-/-}$ mice abolish the production of anti-dsDNA antibodies (Becker-Herman et al., 2011). The recent findings of B cell intrinsic defect (Becker-Herman et al., 2011; Recher et al., 2012) open a new scenario in tolerance breakdown in WAS although the underlying mechanisms are still unclear. It is known that B cell tolerance is established through central and peripheral checkpoints during B cell maturation which require proper BCR and TLR signaling together with extrinsic factors (Meffre, 2011). The cytoskeleton controls the distribution of the BCR and shapes its signaling (Batista et al., 2010). In particular, the density of actin network inversely correlates with the rate of BCR diffusion and the restriction of BCR diffusion limits signaling. Since WASP is required for actin polymerization and cytoskeletal organization in B cells (Facchetti et al., 1998; Westerberg et al., 2005), it is reasonable to speculate that the threshold of activation might be altered in WASP-deficient B cells. In the bone marrow, receptor editing is the major mechanism aimed at eliminating self-reactive B cells during differentiation (Monroe and Dorshkind, 2007; von Boehmer and Melchers, 2010) by editing autoreactive receptors through secondary rearrangements in light chain loci (Halverson et al., 2004). Abnormal receptor editing is involved in the loss of central B cell tolerance (Ng et al., 2004). Interestingly, alterations in the regulation of secondary recombination events have been reported in BTK-, Interleukin-1 Receptor-associated Kinase 4 (IRAK4)-, and MyD88-deficient patients and in a group of Common Variable Immunodeficiency (CVID) patients with expanded autoreactive $CD21^{-/low}$ B cells (Ng et al., 2004; Isnardi et al., 2008; Meffre, 2011). Given the interaction of WASP with BTK (Cory et al., 1996; Sharma et al., 2009), the involvement of MyD88 signaling in B cell tolerance (Becker-Herman et al., 2011) and the increased frequency of $CD21^{-}$ B cells in WAS patients (Park et al., 2005), it would be worth to investigate whether receptor editing is defective also in the absence of WASP. Furthermore, in the periphery, survival of autoreactive B cells is supported by high levels of BAFF and APRIL, members of the TNF superfamily, found to be increased in several autoimmune diseases (Townsend et al., 2010) and lymphopenic conditions (Cassani et al., 2010). This represents an important mechanism involved in the regulation of peripheral human B cell tolerance that would be interesting to investigate in WAS. Finally, a new function as regulator of immune response has been described for B cells and is mainly mediated by the secretion of IL-10 (Matsushita et al., 2008; Yanaba et al., 2009; Watanabe et al., 2010). Although the origin of regulatory B cells is unclear, MZ B cells (Lenert et al., 2005; Evans et al., 2007) seem to have regulatory functions. Thus, considering the reduction of MZ B cells in $Was^{-/-}$ mice (Westerberg et al., 2008; Bosticardo et al., 2011), it would be interesting to investigate whether a defect in regulatory B cell function is a factor contributing to autoimmunity.

In conclusion, together with the defects already described in the literature, these future lines of enquiry underline the greater than expected extent to which the WASP deficiency affects the immune system. Further research is necessary to define the underlying molecular and cellular mechanisms leading to autoimmunity, which represents the main collateral damage caused by WASP deficiency.

ACKNOWLEDGMENTS

This work was supported by grants from Italian Telethon Foundation (to Anna Villa), and Ministero della Salute RF 2007-2008-2009 Giovani Ricercatori Grant (to Marita Bosticardo). Maria Carmina Castiello conducted this study as partial fulfillment of her PhD. in Molecular Medicine, Program in Basic and Applied Immunology, San Raffaele University, Milan, Italy. Figures were produced using Servier Medical Art (www.servier.com).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 April 2012; paper pending published: 06 May 2012; accepted: 01 July 2012; published online: 18 July 2012.

Citation: Catucci M, Castiello MC, Pala F, Bosticardo M and Villa A (2012) Autoimmunity in Wiskott–Aldrich syndrome: an unsolved enigma. Front. Immun. ***3****:209. doi: 10.3389/fimmu.2012.00209*

This article was submitted to Frontiers in Primary Immunodeficiencies, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 27 August 2012
doi: 10.3389/fimmu.2012.00265

Autoimmune dysregulation and purine metabolism in adenosine deaminase deficiency

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Genetic defects in the adenosine deaminase (*ADA*) gene are among the most common causes for severe combined immunodeficiency (SCID). ADA-SCID patients suffer from lymphopenia, severely impaired cellular and humoral immunity, failure to thrive, and recurrent infections. Currently available therapeutic options for this otherwise fatal disorder include bone marrow transplantation (BMT), enzyme replacement therapy with bovine ADA (PEG-ADA), or hematopoietic stem cell gene therapy (HSC-GT). Although varying degrees of immune reconstitution can be achieved by these treatments, breakdown of tolerance is a major concern in ADA-SCID. Immune dysregulation such as autoimmune hypothyroidism, diabetes mellitus, hemolytic anemia, and immune thrombocytopenia are frequently observed in milder forms of the disease. However, several reports document similar complications also in patients on long-term PEG-ADA and after BMT or GT treatment. A skewed repertoire and decreased immune functions have been implicated in autoimmunity observed in certain B-cell and/or T-cell immunodeficiencies, but it remains unclear to what extent specific mechanisms of tolerance are affected in ADA deficiency. Herein we provide an overview about ADA-SCID and the autoimmune manifestations reported in these patients before and after treatment. We also assess the value of the ADA-deficient mouse model as a useful tool to study both immune and metabolic disease mechanisms. With focus on regulatory T- and B-cells we discuss the lymphocyte subpopulations particularly prone to contribute to the loss of self-tolerance and onset of autoimmunity in ADA deficiency. Moreover we address which aspects of immune dysregulation are specifically related to alterations in purine metabolism caused by the lack of ADA and the subsequent accumulation of metabolites with immunomodulatory properties.

Keywords: adenosine deaminase, severe combined immunodeficiency, ADA-SCID, autoimmunity, gene therapy

THE ADA METABOLISM

THE ADA ENZYME

As an enzyme of the purine salvage pathway, adenosine deaminase (ADA) catalyzes the deamination of adenosine and 2′-deoxyadenosine, as well as several naturally occurring methylated adenosine compounds (Hirschhorn and Ratech, 1980; Ratech et al., 1989). The deamination of adenosine and 2′-deoxyadenosine gives rise to inosine and deoxyinosine, respectively (Hirschhorn and Candotti, 2006). Further conversion of these deaminated nucleosides leads to hypoxanthine, which can be either transformed irreversibly into uric acid or salvaged into mononucleosides (**Figure 1**).

Although ADA is present in all cell types, its enzyme activity differs considerably among tissues. The highest amounts in humans are found in lymphoid tissues, particularly the thymus, the brain, and gastrointestinal tract. The ADA enzyme is ubiquitously expressed both intracellularly and on the cell surface where it complexes with two molecules of CD26 as a combined protein (Kameoka et al., 1993).

THE ADA SUBSTRATES ADENOSINE AND 2′-DEOXYADENOSINE

2′-Deoxyadenosine is a component of DNA and primarily derives from its breakdown. Therefore, 2′-deoxyadenosine concentration is expected to be highest at sites of cell death, such as the bone marrow and thymus, where lymphocytes undergo apoptotic death during differentiation and selection. 2′-Deoxyadenosine behaves as a cytotoxic metabolite and is generally considered the primary cause of lymphotoxicity in ADA-severe combined immunodeficiency (SCID; Hirschhorn and Candotti, 2006). The most striking metabolic alteration in ADA deficiency is the accumulation of massive amounts of dATP in erythrocytes and lymphocytes (Hirschhorn et al., 1992). This results from uptake of increasing 2′-deoxyadenosine present in surrounding body fluids with subsequent intracellular phosphorylation and trapping.

Adenosine on the other hand is a component of adenine nucleotides including ATP and RNA (Hirschhorn and Candotti,

Abbreviations: ADA, adenosine deaminase; Adora, adenosine receptor(s); ANA, anti-nuclear antibody; BCR, B cell receptor; BMT, bone marrow transplantation; HLA, human leukocyte antigen; HSC, hematopoietic stem cell; HSC-GT, hematopoietic stem cell gene therapy; PEG-ADA, pegylated bovine ADA; SCID, severe combined immunodeficiency; TCR, T-cell receptor; TLR, Toll-like receptor; Tregs, naturally occurring regulatory T cells.

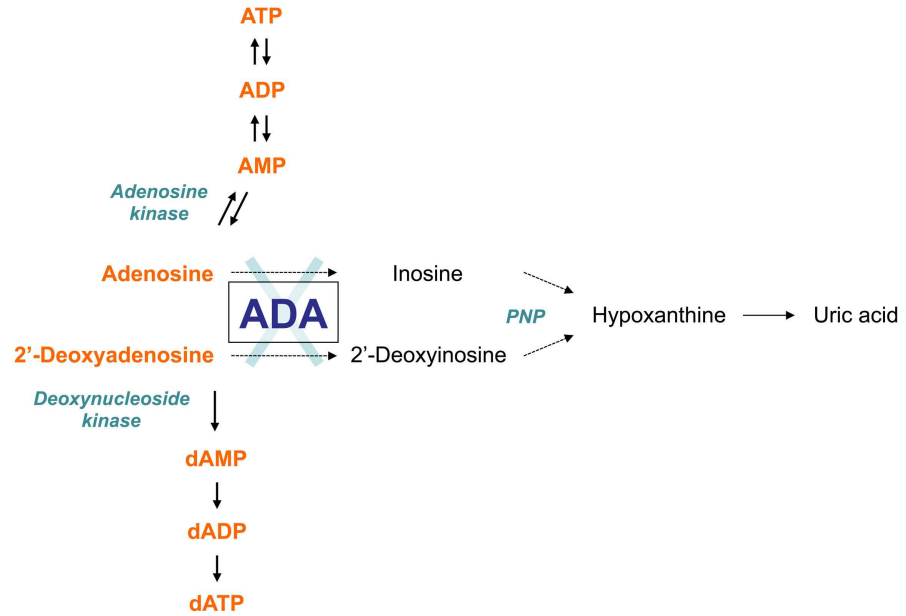

FIGURE 1 | The adenosine deaminase (ADA) metabolism. ADA is an enzyme of the purine salvage pathway, which catalyzes the irreversible deamination of adenosine and 2′-deoxyadenosine into inosine and 2′-deoxyinosine, respectively. Most adenosine derives from endogenous breakdown of ATP and degradation of RNA, or is taken up exogenously by ubiquitously expressed nucleoside transporters. Unlike adenosine, 2′-deoxyadenosine is formed by DNA degradation is predominantly catabolized by ADA. Further conversion of inosine nucleoside leads to hypoxanthine, which can either enter a non-reversible pathway to uric acid or salvaged back into other mononucleosides. In the absence of ADA, the presence of these alternative "bypass" pathways results in normal concentrations of the catabolic products of the enzyme reaction in patients with ADA-SCID. Conversely, the levels of ADA substrates, adenosine and 2′-deoxyadenosine, are not only found in increased amounts in extracellular body fluids, but they also "spill over" into additional pathways normally only minimally utilized, thus contributing to the pathogenic mechanisms of the disease.

2006). Elevated adenosine levels, as occurring in ADA deficiency contribute to apoptosis and block in the differentiation of thymocytes, causing severe T lymphopenia in mice and humans (Apasov et al., 2001; Gaspar et al., 2009; Poliani et al., 2009). Moreover adenosine, acting through cell surface G protein-coupled receptors, functions as an extracellular signal transducer in a variety of physiological processes (Olah and Stiles, 1995). Apart from T-cell receptor signaling (Huang et al., 1997), adenosine is involved in the control of heart rate and blood pressure (Fukunaga et al., 1982; Belardinelli et al., 1989), renal function (Churchill and Bidani, 1982), inflammatory responses (Blackburn, 2003), and in neurotransmission (Fredholm and Dunwiddie, 1988).

ADA-SCID

Adenosine deaminase deficiency is the second-most prevalent form (approximately 20%) of SCID. The overall incidence in Europe is estimated to range between 1:375,000 and 1:660,000 live births. ADA-deficient patients suffer from lymphopenia, severely impaired cellular and humoral immune function, failure to thrive, and a rapidly fatal course due to infection (Hirschhorn and Candotti, 2006). Moreover, autoimmune manifestations are commonly observed in milder forms of the disease. Currently available therapeutic options include bone marrow transplantation (BMT), enzyme replacement therapy with bovine ADA (PEG-ADA), or hematopoietic stem cell gene therapy (HSC-GT).

IMMUNE DEFECTS

Lymphopenia and attrition of immune function over time are the two findings common to all presentations of ADA deficiency. It is associated with thymic hypoplasia and a severe depletion of all three major categories of lymphocytes, T-, B-, and NK-cells (Buckley et al., 1997). Absence of cellular and humoral immunity and a rapidly fatal course due to infections with fungal, viral, and opportunistic agents are characteristic of early onset forms of ADA deficiency (Giblett et al., 1972; Buckley et al., 1997). Total immunoglobulin levels may be only slightly depressed at birth due to the maternal contribution of IgG, whereas both IgM and IgA, which ordinarily do not cross the placental barrier, are often absent. However, once IgG levels decline as maternal antibodies are cleared, a pronounced hypogammaglobulinemia signals the absence of humoral immunity (Morgan et al., 1987; Hirschhorn and Candotti, 2006). About 20% of ADA-SCID cases occur later in childhood (delayed) or beyond (late/adult onset). Delayed or late-onset patients have significant immunodeficiency, but variable clinical manifestations (Ozsahin et al., 1997). These forms show progressive immunological and clinical deterioration, often associated with autoimmune manifestations, including hemolytic anemia, and immune thrombocytopenia (Parkman et al., 1975; Aiuti et al., 2003). Serum immunoglobulin levels are altered in late-onset patients, with IgG2 levels being highly reduced or absent. IgE levels are elevated and often associated to eczema and asthma. An inability to produce antibodies against

polysaccharide and pneumococcal antigens was frequently found in ADA-SCID patients with milder forms of the disease (Levy et al., 1988).

NON-IMMUNE DEFECTS

The initial and most devastating presentation of ADA-SCID is due to the immune defects (Gaspar et al., 2009). Nonetheless, several non-immune abnormalities have been described in ADA deficiency, indicating that this disease should be considered a systemic metabolic disorder (Aiuti et al., 2003; Hirschhorn and Candotti, 2006). ADA is ubiquitously expressed in all cell types; when absent, the systemic metabolic toxicity is frequently associated with organ damage (Sauer and Aiuti, 2009). These include hepatic and renal disease (Bollinger et al., 1996), skeletal alterations (Sauer et al., 2009), neurological abnormalities (Honig et al., 2007; Titman et al., 2008), and behavioral impairments (Rogers et al., 2001). Because complications from infections usually predominate in the clinical presentation of infants with ADA deficiency, the full spectrum of non-immunologic manifestations and their natural course may be obscured (Honig et al., 2007). It is important to note, that several abnormalities have been described in few patients only, and might reflect effects of infectious agents rather than primary defects due to ADA deficiency: i.e., renal and adrenal abnormalities, phyloric stenosis, and hepatic disease (Hirschhorn and Candotti, 2006).

THERAPIES FOR ADA DEFICIENCY

Bone marrow transplantation with allogeneic HSC has long been considered the mainstay of ADA-SCID treatment. However, unlike other SCID forms, two other treatment options are available for ADA-SCID: enzyme replacement therapy with pegylated bovine ADA (PEG-ADA) and autologous HSC-GT (Hershfield et al., 1987; Aiuti et al., 2009). The availability of different treatment modalities presents an opportunity for improved patient care but also difficulties in deciding upon the specific choice of treatment for individual patients (**Figure 2**). Making the correct choice is further complicated by the fact that ADA deficiency is not purely an immune defect, and that the systemic manifestations, which can be of major clinical consequence, must also be managed (Gaspar et al., 2009).

HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic stem cell-transplantation (BMT) from allogeneic human leukocyte antigen (HLA)-compatible sibling donors resulting in long-term survival and effective immune reconstitution is the treatment of choice for patients with ADA-SCID and other severe variants of primary immunodeficiencies. Since less than 20% of ADA-SCID patients have access to HLA-matched family donors, transplants are often performed from mismatched family or matched unrelated donors (Antoine et al., 2003; Gaspar et al., 2009; Ferrua et al., 2010). A recent retrospective analysis on the specific outcome of transplants for ADA-SCID collected data from several multicenter studies and analyzed the survival of 106 patients who received a total of 119 transplants (Hassan et al., 2012). BMT from matched sibling and family donors had a significantly better overall survival (86 and 81%) in comparison to BMT from matched unrelated (66%) and haploidentical donors (43%). Indicating that despite recent progress in transplantation, the use of alternative donors is still associated with a reduced overall survival (Gaspar et al., 2009). This is further complicated by the fact that ADA-SCID patients are more difficult to transplant especially from unrelated and haploidentical donors possibly due to their need for conditioning and the underlying metabolic nature of the disease (Gaspar et al., 2009; Sauer et al., 2009). While superior survival was seen in patients who received unconditioned transplants in comparison to myeloablative procedures (81 and 54%), non-engraftment was a major problem after unconditioned haploidentical transplants (Hassan et al., 2012).

Long-term immune recovery showed that regardless of transplant type, overall T-cell numbers were similar although a faster rate of T-cell recovery was observed following matched sibling or matched unrelated BMT. Humoral immunity and donor B cell engraftment was achieved in nearly all evaluable surviving patients and most patients were able to discontinue immunoglobulin replacement, suggesting that immune recovery is relatively complete (Hassan et al., 2012). According to the available data, the immunological and metabolic recovery after transplant is well maintained even after 10 years or longer in some patients (Gaspar et al., 2009).

Nevertheless delayed or suboptimal immune reconstitution as a result of poor early engraftment or gradual decline in immune functions is observed in a significant fraction of surviving patients (Gaspar et al., 2009). Complications such as graft-versus-host disease, autoimmune and inflammatory manifestations, persistent infections, and disease-related issues have been described (Honig et al., 2007; Titman et al., 2008; Mazzolari et al., 2009).

In summary, the results obtained with transplantation from HLA-identical siblings or family donors indicate superior donor/host compatibility and outcome both in terms of survival and sustained immune recovery. Whereas the current evidence suggests that haploidentical donor transplants (performed with or without conditioning) have a poor chance of success and are therefore only undertaken if no other treatment options are available (Gaspar et al., 2009).

ENZYME REPLACEMENT THERAPY WITH PEG-ADA

Enzyme replacement therapy with PEG-ADA was developed as lifesaving, not curative treatment for patients lacking an HLA-compatible donor. Attachment of PEG through lysine residues confers several therapeutically beneficial properties to ADA (Abuchowski et al., 1977; Davis et al., 1981). This chemical modification of the bovine enzyme reduces its immunogenicity and prevents its degradation by plasmatic proteases as well as the binding of neutralizing antibodies (Abuchowski et al., 1977; Davis et al., 1981). Thereby the circulating life of the compound is prolonged from minutes to days as clearance from the circulation is inhibited (Booth and Gaspar, 2009). Cellular uptake of PEG-ADA is insignificant and its distribution is limited to the plasma. Enzymatically active ADA continuously circulates and eliminates accumulating adenosine and 2′-deoxyadenosine metabolites (Chan et al., 2005). The principle of exogenous PEG-ADA administration is based on the direct conversion of accumulating ADA substrates in the plasma and the indirect reduction of intracellular toxic metabolites by diffusion.

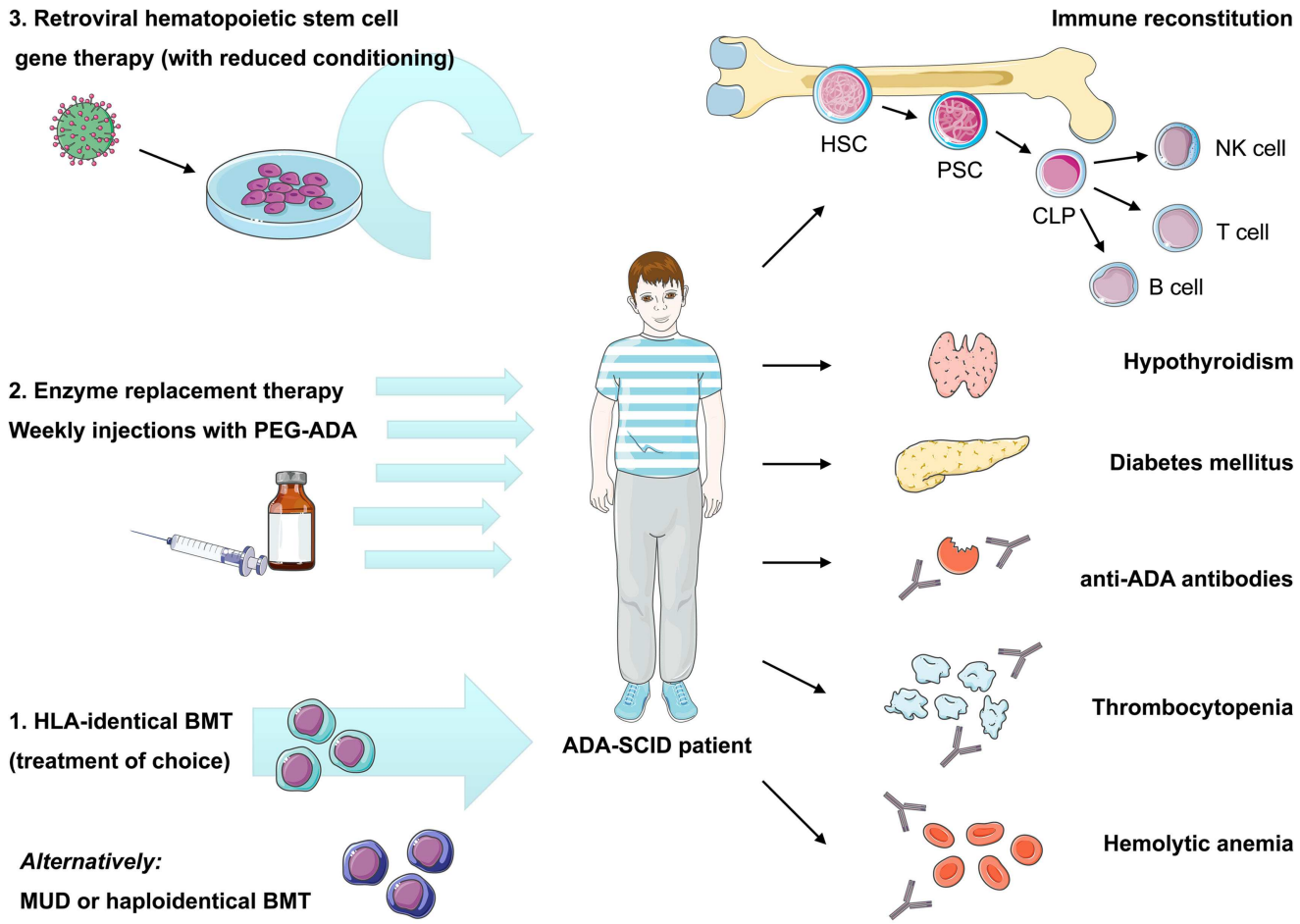

FIGURE 2 | Current therapeutic options in ADA-SCID and reported autoimmune manifestations after treatment. Immune reconstitution in ADA deficiency can be achieved by bone marrow transplantation, enzyme replacement, or gene therapy, nonetheless recovery of immune functions may vary depending on the applied treatment and patient's characteristics. Treatment of choice remains bone marrow transplantation from an HLA-identical sibling donor, while transplants from alternative donors are associated with high morbidity and mortality. Enzyme replacement therapy using pegylated bovine ADA is a non-curative treatment requiring weekly intramuscular injections with PEG-ADA. ADA-SCID has been the pioneer disease for the development of human gene therapy. It is based on the reinfusion of autologous HSC transduced with a retroviral vector containing the *ADA* cDNA. Variable degrees of immune reconstitution can be achieved by these treatments, but onset of autoimmunity is of concern in post-treatment ADA-SCID patients. Reported autoimmune manifestations include: autoimmune hypothyroidism, diabetes mellitus, thrombocytopenia, hemolytic anemia, and development of anti-ADA antibodies. HLA, human leukocyte antigen; BMT, bone marrow transplantation; MUD, matched unrelated donor; PEG-ADA, pegylated bovine ADA; HSC, hematopoietic stem cell; PSC, pluripotent stem cell; CLP, committed lymphocyte precursor; NK, natural killer cell.

To date more than 150 patients worldwide have received this treatment (Booth and Gaspar, 2009; Gaspar et al., 2009). PEG-ADA is usually administered weekly or bi-weekly by intramuscular injections throughout life. In general, PEG-ADA treatment seems to be well tolerated, with clinical benefits appreciable after the first month of therapy (**Figure 3**). Studies have shown that upon the initiation of PEG-ADA therapy, the absolute numbers of circulating T- and B-lymphocytes and NK-cells increase and protective immune function develops (Weinberg et al., 1993). Although only limited information is available, some analysis indicated that about half of PEG-ADA treated patients discontinued IVIg (Gaspar et al., 2009), whereas long-term follow-up suggests that immune recovery is often incomplete (Booth and Gaspar, 2009). Two retrospective studies showed that despite initial improvements, the lymphocyte counts of all PEG-ADA treated patients were below the normal range at all times. A gradual decline of mitogenic proliferative responses occurred after a few years of treatment and normal antigenic responses occurred less than expected (Kohn, 2008; Serana et al., 2010). No toxic or hypersensitivity reactions have been reported with PEG-ADA administration. However, several other side effects have been reported including manifestations of immune dysregulations including autoimmunity (type I diabetes, hypothyroidism, immune thrombocytopenia, hemolytic anemia) and allergic manifestations (Notarangelo et al., 1992; Ozsahin et al., 1997). An additional concern with PEG-ADA beyond about 8–10 years is the emergence of serious complications, including lymphoid and hepatic malignancies, and progression of chronic pulmonary insufficiency (Gaspar et al., 2009).

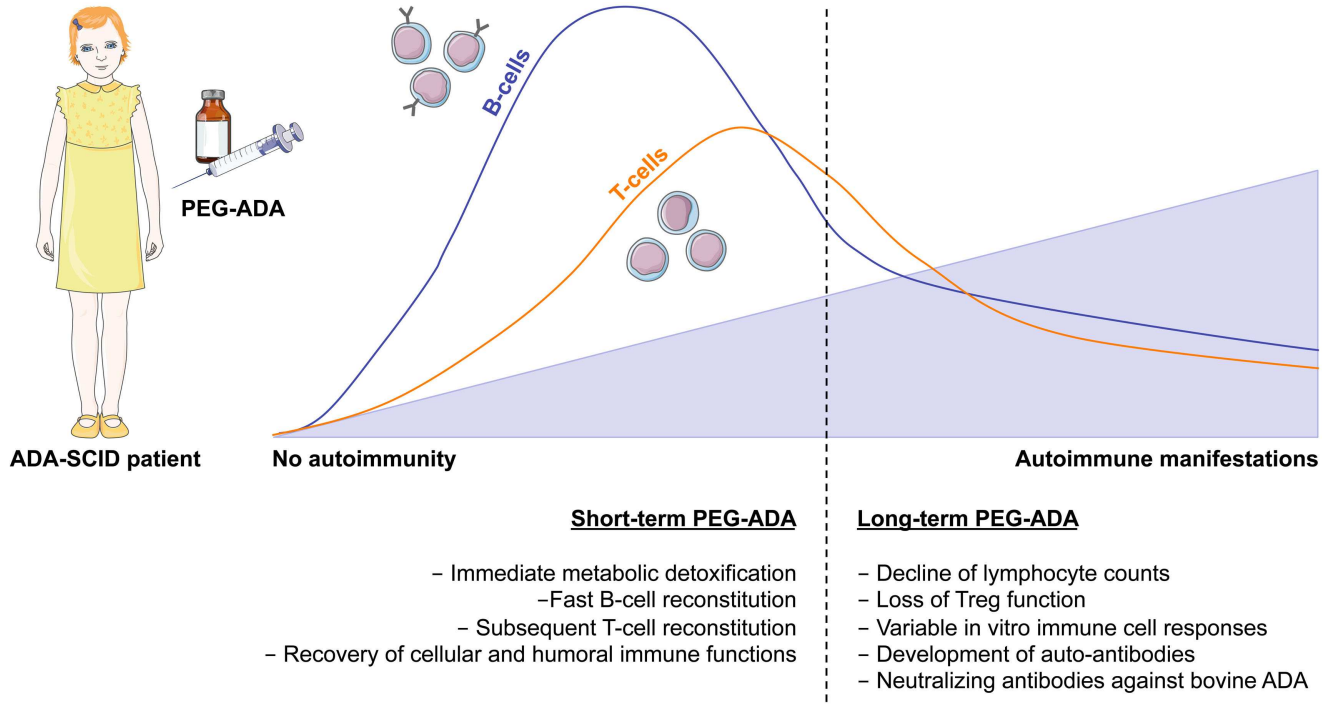

FIGURE 3 | Immune reconstitution and development of autoimmunity after PEG-ADA treatment. Enzyme replacement therapy with pegylated bovine ADA is a lifesaving but non-curative treatment for ADA-SCID patients. It provides metabolic detoxification and protective immune function with patients remaining clinically well, but immune reconstitution is often suboptimal and may not be long-lived. Shortly after initiation of PEG-ADA treatment, patients show recovery of B-cell counts, followed by a gradual increase in T-cell numbers and reconstitution of immune cell functions. However, the long-term consequences of PEG-ADA treatment are unknown. Immune recovery in B and T- cells is below normal levels. Major concerns are the susceptibility to opportunistic infections and the development of autoimmunity due to lymphopenia with gradual decline of immune functions and perturbation of T- and B-cell tolerance.

The main side effect associated with the use of PEG-ADA is the development of anti-ADA antibody. The development of specific IgG antibody to bovine peptide epitopes of PEG-ADA has been reported by several groups and often coincides with an improvement in humoral immunity (Chaffee et al., 1992; Lainka et al., 2005; Booth et al., 2007). In about 10% of treated patients, inhibitory antibodies lead to the enhanced clearance of PEG-ADA with subsequent decline in metabolic parameters and immune function (Chaffee et al., 1992; Hershfield, 1995; Lainka et al., 2005).

GENE THERAPY

Hematopoietic stem cell gene therapy is a promising therapeutic option for genetic disorders of the immune system (Bordignon and Roncarolo, 2002; Fischer et al., 2005). ADA-deficient SCID has been under intensive preclinical and clinical investigation and nowadays represents a paradigmatic model of gene therapy for inherited disorders (Aiuti et al., 2003, 2007). The strong rationale for somatic gene therapy and the need for alternative treatments led to the design of clinical trials based on retroviral-mediated gene transfer of the normal *ADA* gene into autologous HSCs (Aiuti, 2004). Replication-deficient, recombinant retroviruses derived from the backbone of Moloney murine leukemia virus (MLV) were selected for these trials because of the available long-term experience and their ability to efficiently insert the therapeutic gene into the genome of dividing hematopoietic cells.

Since 2000, 37 patients have been treated in Italy, UK, and USA, achieving substantial clinical benefit in the majority of them. All patients received reduced intensity conditioning and PEG-ADA was discontinued to exploit the selective growth advantage for gene corrected over defective cells. At present, all patients are alive and in 26 patients PEG-ADA is no longer required (Aiuti et al., 2009; Gaspar et al., 2011; Montiel-Equihua et al., 2012). Gene therapy resulted in sustained engraftment of transduced cells, increased lymphocyte counts, improvement of cellular and humoral responses, and effective metabolic detoxification (Aiuti et al., 2009; Gaspar et al., 2011). Gene corrected cells were detected in myeloid and lymphoid subsets, the latter being more represented due to their survival advantage (Aiuti et al., 2009; Gaspar et al., 2011). In the HSR-TIGET study, all children maintained stable engraftment of vector ADA-transduced CD34+ cells with sustained systemic detoxification (Aiuti et al., 2009). At present, 15 of the 18 treated children do not require enzyme replacement therapy, with the longest follow-up at 11 years after treatment (Aiuti et al., 2009; Ferrua et al., 2010). These findings demonstrated the clinical efficacy of *ADA* gene transfer in restoring normal immune function and metabolic functions of ADA-SCID patients.

Unlike trials with gammaretroviral vectors in other diseases like X-linked SCID (Hacein-Bey-Abina et al., 2008; Howe et al., 2008), chronic granulomatous disease (Ott et al., 2006) and Wiskott–Aldrich Syndrome (Trobridge, 2011), the cumulative experience

of these studies for ADA-SCID (Aiuti et al., 2009; Ferrua et al., 2010; Montiel-Equihua et al., 2012) did not reveal leukemic or oncogenic events, indicating that ADA-SCID gene therapy has a favorable risk/benefit profile. Unique risk factors may have contributed to the differential outcome of the other trials, such as vector constructs or promoters, inappropriate expression of transgenes involved in cell signaling (Kohn, 2008), cooperation between transgene and cellular oncogenes (Dave et al., 2009), or the disease background itself (Shou et al., 2006).

AUTOIMMUNITY IN ADA-SCID

Immunodeficiency and autoimmune phenomena may occur concomitantly in the same individual (Etzioni, 2003). Immune dysregulation, which often manifests as multiple forms of autoimmunity, can affect both the adaptive and innate immune system, indicating that all these immune components are required for the appropriate development of tolerance in humans (Cunningham-Rundles, 2011). Since varying degrees of immune reconstitution can be achieved by the available treatment options for ADA-SCID, breakdown of tolerance and development of autoimmunity can represent a major concern. Autoimmune dysregulation are frequently observed in patients with milder forms of the disease or late-onset patients. They may manifest as autoimmune hypothyroidism, diabetes mellitus, hemolytic anemia, and immune thrombocytopenia (Notarangelo et al., 1992; Ozsahin et al., 1997; **Figure 2**).

Similar complications, such as autoimmune hemolytic anemia and autoimmune thyroiditis, have also been reported in at least nine patients after long-term PEG-ADA treatment (Ratech et al., 1989; Notarangelo et al., 1992; Ozsahin et al., 1997; Gaspar et al., 2009; Serana et al., 2010). Refractory hemolytic anemia was fatal in three patients (Gaspar et al., 2009). Two additional studies assessed defects in the lymphoid compartments of ADA-SCID patients following PEG-ADA. Different degrees of abnormalities in the B-cell compartment and inability to respond to vaccines, despite the presence of normal serum-Ig or hypogammaglobulinemia were reported (Malacarne et al., 2005). Moreover, a retrospective longitudinal analysis in ADA-SCID patients treated with PEG-ADA showed that decreased levels of newly produced B cells underlie the progressive and significant decrease in circulating B cells in these patients (Serana et al., 2010). Since long-term PEG-ADA treatment is associated with abnormalities in B cell subsets, but often also with a decrease in T-cell functions (Malacarne et al., 2005), a limited B or T-cell repertoire combined with alterations in peripheral tolerance could further favor breakdown of tolerance (**Figure 3**).

No specific reports on immune dysregulation or autoimmunity in BMT-treated ADA-SCID patients are available in literature (Serana et al., 2010). Nevertheless, autoimmune manifestations have been reported in larger single-center studies on BMT-treated patients with various kinds of immunodeficiencies, including ADA deficiency (Mazzolari et al., 2009; Neven et al., 2009). The major immune dysregulations observed in both studies included thyroid autoimmunity, autoimmune hemolytic anemia, and glomerulonephritis (Mazzolari et al., 2009; Neven et al., 2009).

Most recently autoimmune manifestations have also been described in patients treated with HSC-GT (Aiuti et al., 2009). Four ADA-SCID patients, including one patient that already showed immune dysregulation while on PEG-ADA, developed signs of autoimmunity, such as hemolytic anemia, thrombocytopenia, autoimmune hepatitis, and autoimmune thyroiditis (Aiuti et al., 2009 and unpublished observation).

ADA-DEFICIENT MOUSE MODEL

The availability of a genetic animal model for ADA deficiency allowed a wide range of biochemical and immunological experiments that are not feasible in humans. The first attempts to generate ADA-deficient mice lead to their perinatal death due to severe liver damage (Blackburn et al., 1998). Subsequent studies suggested that ADA expression in trophoblast cells of the placenta is critical for fetal development in the mouse. Thus, ADA-deficient mice were successfully generated by specifically targeting expression of an *ADA* minigene to the trophoblast lineage of ADA+/− mice and by inter-crossing these mice. This gave rise to litters that contained mice expressing the *ADA* minigene in their placenta that were also homozygous for the ADA null allele (ADA−/−; Blackburn et al., 1998).

UNTREATED ADA−/− MICE

The ADA−/− mouse reproduces not only the biochemical but also the immunological abnormalities of the human disease phenotype. They manifest both combined immunodeficiency as well as metabolic abnormalities and are therefore commonly used to assess the effect of ADA deficiency not only on the lymphoid organs and peripheral blood, but also its systemic organ toxicity. ADA−/− deficient mice die at approximately 3 weeks of age from severe respiratory distress (Blackburn et al., 1998).

Initial examinations of the thymus and spleen revealed a substantial decrease in organ size. The cellular proportion from the thymus of ADA−/− mice showed a significant increase in the percentage double-negative immature thymocytes, accompanied by a decrease in the percentage of CD4+ or CD8+ single-positive thymocytes. T-cell apoptosis was abundant in the ADA-deficient thymi (Blackburn et al., 1998). ADA−/− splenic B lymphocytes showed defects in proliferation and activation with high propensity to undergo B cell receptor-mediated apoptosis. As a result, profound loss of germinal center architecture was noted, which may be responsible for impaired B cell development (Aldrich et al., 2003). Lymphopenia was also seen in the peripheral circulation, confirming that this model of ADA deficiency exhibits a SCID phenotype.

At death, the severe immune deficiency and organ alterations are the most prominent features, whereas no apparent autoimmune manifestations can be observed. The almost complete absence of effector T- and B-cell populations in these mice and the high levels of anti-inflammatory adenosine might prevent their development in the first 3 weeks of life. Reconstitution of effector T- and B-cells as well as metabolic detoxification after treatment might therefore be requirements for the onset of autoimmunity (Sauer et al., 2012a).

MODEL FOR AUTOIMMUNITY IN ADA-DEFICIENT MICE

Similarly to ADA-SCID patients, ADA−/− mice can be treated with PEG-ADA, HSC-GT with transduced BM ADA−/− cells, or BMT with wild type donor cells (Mortellaro et al., 2006; Sauer

et al., 2009). A dose of 1000 U/kg/week of PEG-ADA starting from postnatal day 10 provides rescue and metabolic detoxification in ADA−/− mice (Blackburn et al., 2000). HSC-GT is performed using a SIN-lentiviral vector driving ADA expression from the phosphoglycerate kinase (PGK) promoter (Mortellaro et al., 2006), instead of the gammaretroviral vector used in the clinical trial. A long-term comparative approach between these three treatment options revealed important new information on their efficacy and established a model for autoimmunity in the context of long-term PEG-ADA treatment (Sauer et al., 2012a).

The long-term survival of PEG-ADA, HSC-GT, and BMT-treated mice was comparable between the three groups (60–70% with respect to wildtype). This outcome was the result of an early mortality in the BMT and HSC-GT treated groups, while PEG-ADA treated mice had a less stable long-term survival. As expected from the fact that PEG-ADA remains in circulation without entering in cells, ADA activity in PEG-ADA treated mice was exclusively detectable in the plasma. Reconstitution of enzymatic activity in RBC, BM, spleen, and thymus from BMT-treated mice was comparable to wildtype, while only slightly lower in HSC-GT treated mice (Sauer et al., 2012a).

Strikingly, ADA−/− mice treated with PEG-ADA developed multiple autoantibodies and hypothyroidism in contrast to mice treated with HSC-GT or BMT. Proliferation of various lymphocyte subpopulations, including B cells and highly abnormal antibody production affecting all types of antibody subclasses was observed in PEG-ADA treated mice. Moreover, autoantibodies that reacted to ADA, platelets, the thyroid, and the gastrointestinal tract were detected in the sera from PEG-ADA treated mice. Focal atresia with non-secreting follicles, an increase in apoptotic cells in affected tissue areas and significantly elevated levels of thyroid-stimulating hormone (TSH) represent signs of autoimmune hypothyroidism. The role of autoantibodies against the stomach and intestine developing in PEG-ADA treated mice, without causing gross pathological alterations, remained unclear. However, it was hypothesized that the occurrence of antibody responses to GI tissues not only interferes with nutrient uptake, but also reflect alterations in gastrointestinal immunity (Sauer et al., 2012a). The established mouse model for autoimmunity after PEG-ADA treatment represents a valuable model for future studies on the *in vivo* effects of PEG-ADA on immune cell function and inflammatory responses.

Interestingly, PEG-ADA treated mice produced antibodies to ADA, platelets, the thyroid, and gastrointestinal tract, but not other organs such as the pancreas or endocrine glands. The strong overlap of autoimmune manifestations observed in this model of autoimmunity in ADA−/− mice with those reported in ADA-deficient patients suggests that a component of autoimmune susceptibility may map to the target tissue. In both humans and in mouse models, single genetic loci have been linked with susceptibility to multiple autoimmune diseases. The genes underlying such loci, including AIRE, FoxP3, CTLA-4, and PTPN22, are likely to confer a general predisposition to the failure of immune tolerance and development of an auto-aggressive immune response (Hill et al., 2007). However, other loci are clearly disease specific, and presumably modify a generalized predisposition to confer organ/disease specificity. Interestingly, recent studies have implicated ADA polymorphisms in the development of type1 diabetes and rheumatoid arthritis (Sebastiani et al., 2006; Saccucci et al., 2009).

ROLE OF ADA METABOLITES IN IMMUNE CELL DEVELOPMENT AND FUNCTION

Although autoimmunity is frequently observed in certain immunodeficiencies, there is accumulating evidence that ADA deficiency predisposes to this phenomenon not only through general mechanisms of immune dysregulation but also through specific alterations caused by the accumulating ADA metabolites. Main feature of ADA deficiency is the gradual accumulation of adenosine and 2′-deoxyadenosine nucleosides. In the absence of ADA, these nucleosides are metabolized differently into AXP or dAXP, respectively, and exert distinct biochemical action (AXP: AMP, ADP, or ATP; dAXP: dAMP, dADP, or dATP). Several pathophysiological mechanisms have been proposed to describe the role of ADA substrates in cytotoxicity as well as their immunomodulatory properties in patients and in the ADA-deficient mouse model (Hirschhorn and Candotti, 2006). The major effects of adenosine, 2′-deoxyadenosine and their nucleotide byproducts are summarized in **Table 1**.

CYTOTOXICITY OF 2′-DEOXYADENOSINE AND dATP

Based upon *in vivo* and *in vitro* findings, several mechanisms are believed to account for the block of lymphocyte development in ADA-SCID (Hirschhorn and Candotti, 2006). The biochemical hallmarks of ADA deficiency consist of the general belief, that 2′-deoxyadenosine is the primary cause of lymphotoxicity in ADA-SCID, which exerts its effects at the nucleoside level or after conversion to dATP. Although 2′-deoxyadenosine is a weak substrate for adenosine kinase and deoxycytidine kinase, in the absence of ADA these enzymes can phosphorylate 2′-deoxyadenosine. In turn, the resulting dATP pool expansion may interfere with a number of critical metabolic pathways.

These ADA substrate accumulations inhibit methyl-transfer reactions by suicide inactivation of *S*-adenosylhomocysteine (SAH) hydrolase (Hershfield et al., 1979). dATP is known to be a feedback inhibitor of ribonucleotide reductase. Its inhibition causes an imbalance of deoxynucleotides (dNTP), leading to an impairment of DNA synthesis, which is critical for the expansion of lymphocytes in response to antigenic challenge (Benveniste et al., 1995).

ROLE OF ADENOSINE AS ANTI-INFLAMMATORY MEDIATOR

By binding to G-coupled adenosine receptors present on the surface of target cells, adenosine acts as an extracellular signal transducer to exert suppressive functions (Sitkovsky et al., 2004). Physiologically, adenosine-mediated triggering of these receptors can promote a fine-tuning of the inflammatory responses. In the context of defective ADA metabolizing enzyme, where the extracellular levels of adenosine are increased, this regulation may be exaggerated and cause immune dysfunction. This process is mostly regulated by the aberrant engagement of adenosine 2a receptor (Adora2a)-mediated signaling.

Functional studies on T cells from ADA-deficient mice and patients showed an increased susceptibility to apoptosis as well as altered intra- and extracellular signaling leading to impaired T-cell

Table 1 | Effects of ADA metabolites on lymphocyte development and function.

ADA metabolite	Cell type	Mouse/ human	Mode of action	Reference
2′-Deoxyadenosine	Lymphocytes	Human	Inhibition of SAHH activity results in accumulation of *S*-adenosylhomocysteine and inhibition of transmethylation reactions	Hershfield et al. (1979) and Benveniste et al. (1995)
	Fibroblasts	Mouse	SAHH acts as a physiological modulator of Fas-mediated cell death	Ratter et al. (1996)
	T cells	Human	Inhibition of T-cell activation by aberrant Adora2a signaling and PKA hyperactivation	Cassani et al. (2008)
dATP	Lympocytes and RBC	Human	Intracellular ATP depletion	Siaw et al. (1980), Simmonds et al. (1982), Koller et al. (1984), and Simmonds et al. (1984)
	T cells	Human	Inhibition of ribonucleotide reductase causes an imbalance of dNTPs and an impairment of DNA synthesis	Waddell and Ullman (1983), Benveniste et al. (1995)
	T cells	Human	Accumulation of DNA single strand breaks	Cohen and Thompson (1986) and Gangi-Peterson et al. (1999)
	T cells	Mouse	Inhibition of thymocyte development past the CD4−/CD8− double-negative stage	Van de Wiele et al. (2002) and Van de Wiele et al. (2006)
	B cells	Human	Nucleotide pool imbalance affects TdT activity during V(D)J recombination in the bone marrow	Gangi-Peterson et al. (1999)
Adenosine	T cells	Human	Compromised TCR/CD28-driven proliferation and cytokine production, defective activation of NF-κB transcriptional events	Cassani et al. (2008)
	Resting T cells	Human	Upregulation of CD152, CTLA-4, normally involved in the termination of immune responses	Vendetti et al. (2002)
	T cells	Mouse	Decreased TCR-triggered activation and upregulation of activation markers	Apasov and Sitkovsky (1999), Apasov et al. (2001)
	Activated T cells	Mouse	Inhibition of IL-2, TNFα, and INFγ secretion	Erdmann et al. (2005) and Lappas et al. (2005)
	B cells	Human	Adora2a signaling interferes with BCR- and TLR-function, inhibition of B-cell activation after stimulation	Sauer et al. (2012b)
	B cells	Mouse	Profound loss of GC, susceptibility to apoptosis, defects in B-cell proliferation and activation, block in Ag-dependent B-cell maturation	Aldrich et al. (2003)
	B cells	Mouse	Increase of intracellular cAMP suppresses the activation of NF-κB after BCR and TLR-stimulation	Minguet et al. (2005)
	Tregs	Mouse/ human	Adora signaling causes alterations in the CD39/CD73 adenosinergic machinery, upregulation of FoxP3	Sauer et al. (2012a)
	Adaptive Tregs	Mouse	Adora2a signaling inhibits the generation of adaptive effector T cells and promotes the induction of adaptive Tregs, upregulation of FoxP3	Zarek et al. (2008)
ATP	T cells	Human	Purinergic stimulation through P2X receptors prolongs TCR-initiated activation and IL-2 secretion	Yip et al. (2009)
	T cells	Mouse	Antagonism of P2X blunts TCR-mediated activation and results in unresponsiveness to subsequent stimulation	Schenk et al. (2008)
	Tregs	Mouse	Activation of P2X7 inhibits the suppressive potential and stability of Tregs	Schenk et al. (2011)

Adora2a, adenosine 2a receptor; ATP, adenosine-5′-triphosphate; BCR, B cell receptor; cAMP, 3′,5′-cyclic adenosine monophosphate; dATP, 2′-deoxyadenosine 5′-triphosphate; dNTPs, deoxynucleotides; GC, germinal center; INFγ, interferon gamma; NF-κB, nuclear factor kappa B; P2X/P2X7, purinergic receptors; PKA, cAMP-dependent protein kinase A; RBC, red blood cells; SAHH, S-adenosylhomocysteine hydrolase; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase; TNFα, tumor necrosis factor alpha; Tregs, naturally occurring regulatory T cells.

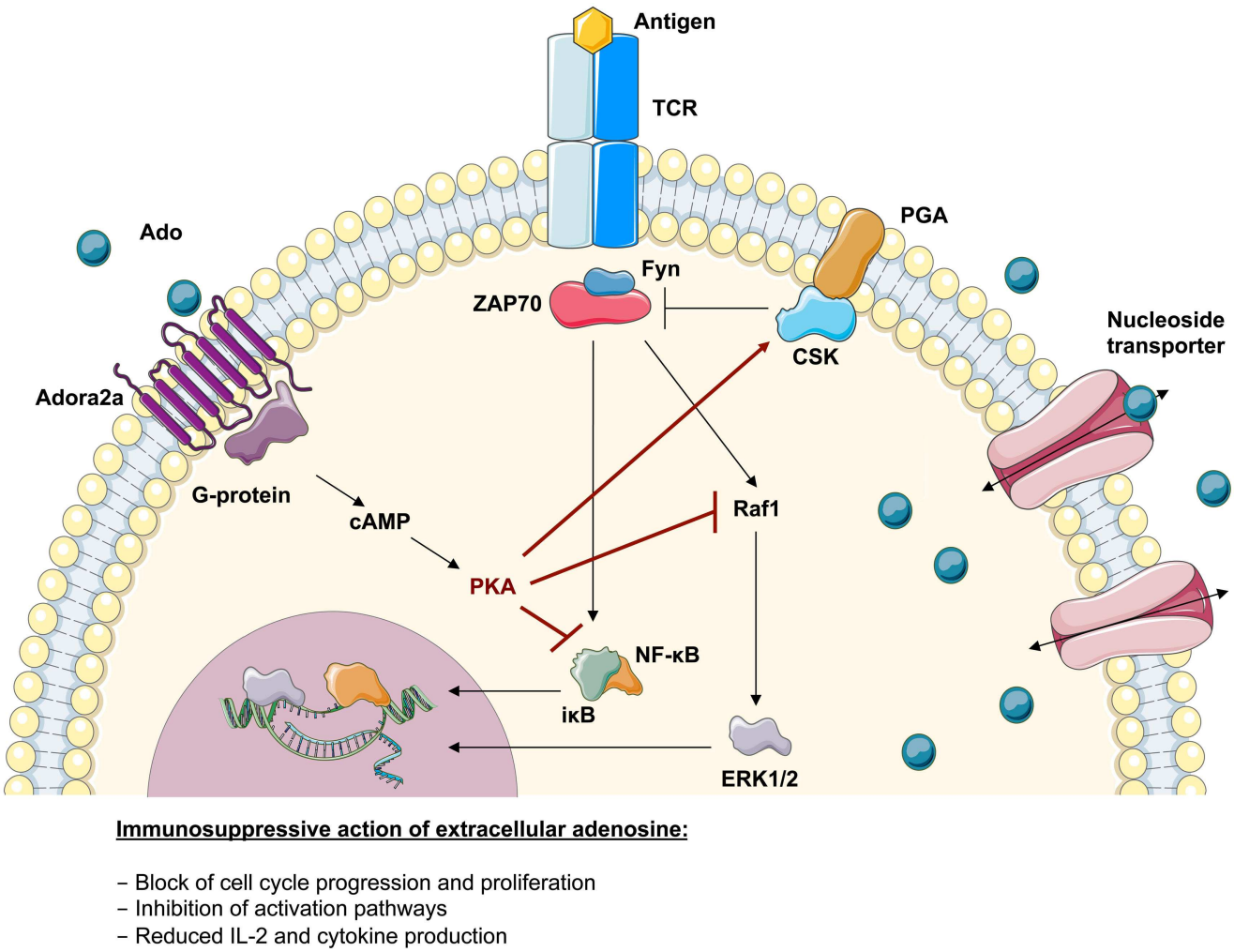

FIGURE 4 | Immunosuppressive action of adenosine in lymphoid cells. Upon TCR-antigen-binding and T-cell activation, the translocation of activated transcription factors and the expression of anti-apoptotic factors supports their survival, proliferation, and differentiation. The adenosine 2a receptor (Adora2a) present on T-lymphocytes interacts with coupled G-proteins to stimulate cAMP formation. The effects of cAMP in T cells are almost entirely mediated by the cAMP-dependent protein kinase A (PKA). It has been shown that the inhibitory properties of PKA are mediated via activation of Csk or inhibition of Raf1. Consistently it has been shown that the severely compromised effector functions in ADA-deficient T cells associates with an intrinsically reduced ERK1/2 signaling, a decreased phosphorylation of IκB and an altered nuclear recruitment of NF-κB. This suggests that the accumulating adenosine levels in an ADA-deficient environment can directly antagonize biochemical events arising from TCR engagement.

function (Apasov and Sitkovsky, 1999; Apasov et al., 2001; Cassani et al., 2008). As summarized in **Figure 4**, the TCR-dependent activation defect in ADA deficiency is augmented by the immunosuppression through extracellular adenosine receptor triggering. Extracellular adenosine induces increased levels of cAMP in T-lymphocytes, which inhibits both proximal signaling events after TCR triggering as well as other downstream effector functions (Huang et al., 1997; Lappas et al., 2005; Ohta et al., 2009). In accordance with previous data obtained in thymocytes from ADA−/− mice (Apasov et al., 2001), IκBα phosphorylation after TCR triggering was low or undetectable in ADA-deficient cells (Cassani et al., 2008). Reduced levels of IκBα phosphorylation and degradation leads to low levels of NF-κB translocation and transcription of target genes in the nucleus, thereby contributing to the functional impairments of ADA-deficient T cells.

Less information is available about the effects exerted by adenosine on B-cell function. Similarly to the alterations in T cells described above, adenosine receptor ligation in B cells inhibits downstream responses to antigen receptor engagement like BCR-induced IκB phosphorylation and the NF-κB pathway after BCR or TLR4 stimulation (Minguet et al., 2005). Adenosine may thereby drive BCR-stimulated B cells toward an anergic rather than an immunogenic response. Recent findings showing defects in BCR and TLR signaling as well as in tolerance checkpoint control in human B cells are discussed and illustrated separately in section "Defects in B-cell tolerance in ADA-SCID."

Overall, these evidences strongly suggest that rather than controlling a single pathway downstream of the TCR or BCR, the immune defects in ADA-deficient lymphocytes may involve multiple pathways converging toward the defective induction

of lymphocyte activation. They also illustrate how extracellular adenosine levels can interfere with the downstream signaling transduction upon activation, thereby exerting its immunosuppressive activity on the transcriptional machinery. Because of cell-specific expression and regulation, aberrant adenosine receptor-mediated signaling might also contribute to the occurrence of autoimmune manifestations observed in some ADA-SCID patients (Kohn, 1996; Ozsahin et al., 1997).

ROLE OF ATP AND OTHER PURINERGIC RECEPTORS

Stimulation of almost all mammalian cell types leads to the release of cellular ATP and autocrine feedback through a diverse array of purinergic receptors (Junger, 2011). ATP binds to two classes of purinergic P2 receptors in the plasma membrane of eukaryotic cells: P2X receptors, which are ligand-gated ion channels, and heterotrimeric G protein-coupled P2Y receptors (Schenk et al., 2011). Depending on the types of purinergic receptors that are involved, autocrine signaling can promote or inhibit immune cell activation and fine-tune adaptive immune responses (Junger, 2011; Schenk et al., 2011).

In addition to the autocrine feedback mechanisms that regulate the function of healthy immune cells, purinergic receptors allow immune cells to recognize ATP that is released from damaged or stressed host cells. Thus, the purinergic signaling systems of immune cells serve an important function in the recognition of danger signals. ATP that is released by stressed cells guides phagocytes to inflammatory sites and promotes clearance of damaged and apoptotic cells (Elliott et al., 2009; Junger, 2011).

To date, little information is available on alterations in the ATP-induced regulation of immune cells in ADA deficiency. It is reported that dATP accumulation in the absence of ADA leads to a cellular depletion of ATP (Siaw et al., 1980; Simmonds et al., 1982, 1984; Koller et al., 1984). The pool of extracellular ATP on the other hand might well be augmented in ADA-deficient lymphoid organs, due to the increased percentage of cells undergoing apoptosis. It can therefore be hypothesized that alterations in ATP concentrations in ADA deficiency also influence T-cell responses on the level of TCR induced activation and in response to stimuli from an inflammatory microenvironment.

BREAK OF TOLERANCE AND CONTRIBUTION OF LYMPHOCYTES TO AUTOIMMUNITY IN ADA DEFICIENCY

Adaptive immunity requires sophisticated regulatory mechanisms to ensure protection to a variety of pathogenic microbes while maintaining immune self-tolerance and preventing autoimmunity (Sakaguchi et al., 2008). The main mechanisms for the induction and maintenance of a self-tolerant repertoire, which is diverse in antigen recognition, are central and peripheral tolerance. Central tolerance is the mechanism able to eliminate newly developing T cells and B cells that have high affinity to self (Mathis and Benoist, 2004). Central tolerance is distinct from peripheral tolerance in that it occurs while cells are still present in the primary lymphoid organs, whereas emigrant cells are controlled through peripheral tolerance mechanisms, after they reach the periphery (Wardemann and Nussenzweig, 2007; Klein et al., 2009). These include suppression of autoreactive cells by regulatory T cells and the generation of hyporesponsiveness (anergy) in lymphocytes, which encounter antigen in the absence of the co-stimulatory signals that accompany inflammation (Meffre and Wardemann, 2008).

Numerous mechanisms have been proposed to explain the break of tolerance and development of autoimmune manifestations, such as defective negative selection of autoreactive T-lymphocytes in the thymus, alterations in the number and/or function of regulatory T cells, defects of the central and peripheral B-cell tolerance checkpoints, impaired apoptosis of autoreactive lymphocytes, break of tolerance due to increased or decreased clearance of apoptotic cells and pathogens, or increased homeostatic lymphoid proliferation and cytokine secretion associated with lymphopenia (Carneiro-Sampaio and Coutinho, 2007; Westerberg et al., 2008; Notarangelo, 2009; Meffre, 2012).

T-CELL TOLERANCE

Central T-cell tolerance mechanisms are based on the elimination or negative selection of the majority of T cells recognizing self with high affinity for negative selection in the thymus. Nonetheless thymic selection is not a tight process and T cells expressing low-avidity TCR on their cell surface are frequently released in the periphery, where they are potentially dangerous to the host as they can be effectively recruited into an autoimmune response (Parish and Heath, 2008).

A major cause of tolerance breakdown is associated with lymphopenia (Daikeler and Tyndall, 2007). This typical state of primary immunodeficiencies may contribute to the induction of spontaneous homeostatic proliferation of residual T cells allowing peripheral expansion of autoreactive cells with a skewed repertoire. Particularly, after conditioning or transplantation these cells may persist, since insufficient thymic reconstitution may affect the control of self-reactivity due to defective negative selection in the thymus and/or reduced regulatory T-cell development and function (Hauri-Hohl et al., 2007). In the case of ADA deficiency, it has been hypothesized that the structure and functions of the thymic microenvironment might be altered, either directly, by toxicity of purine metabolites, or indirectly, by failure of T cells arrested in their development to deliver supportive signals to the thymic stroma (Apasov et al., 2001).

Peripheral tolerance depends on the balance between immune responses to invading pathogens and immune tolerance to self-antigens. In the context of tissue damage and frequently occurring infections in primary immunodeficient patients, apoptotic cells represent a major source of autoantigen. Since apoptosis plays a major role in the deletion of autoreactive lymphocytes and the removal of virus-infected cells, defects in cell death have been implicated in the development of autoimmune diseases and persistent viral infection (Utz et al., 2000). The release of self-antigen into the intracellular space and their presentation mediated by dendritic cells or other antigen-presenting cells may prime naive autoreactive T cells, which were not eliminated by depletion or anergy (Waldner et al., 2004). Several mechanisms exist, including a spectrum of CD4+ regulatory T cells (Tregs), to suppress self-reactive T cells that escape thymic clonal deletion and attenuate anti-pathogen effector mechanisms from inducing immune pathology (Piccirillo and Thornton, 2004). There is ample evidence that Tregs actively mediate suppression to control immune

responses to self- and non-self-antigens and the onset of autoimmunity (Bach, 2003; Sakaguchi, 2005). Lessons from other primary immunodeficiencies have provided unequivocal evidence for the essential role of Tregs in suppressing autoreactive T cells in the periphery (Westerberg et al., 2008). Rising of autoimmunity may not only be linked to a reduction in Treg numbers but also to attenuation of their suppressive activity (Sakaguchi et al., 2008). While this is principally mediated by cell–cell contact, recent findings revealed additional mechanisms of Treg-mediated suppression, including secretion of immunosuppressive cytokines, functional modification or killing of APC, and metabolic disruption (Vignali et al., 2008). Moreover, extracellular adenosine produced by Tregs, has been identified as one of the mechanisms mediating their suppressive activity (Sitkovsky et al., 2008; Mandapathil et al., 2010). Treg cells possess a unique biochemical signature amongst T cells in that they generate and sustain high adenosine concentrations. Since Tregs primarily mediate peripheral control of autoreactive T cells, it is conceivable that this compartment might be specifically affected in ADA-SCID (see also Defective Regulatory T Cell Function in ADA Deficiency). Consequently the autoimmune manifestations associated with ADA deficiency might be the result of an altered purine metabolism interfering with normal regulatory T-cell function (Sauer et al., 2012a).

B-CELL TOLERANCE

A variety of mechanisms ranging from clonal deletion to functional inactivation by anergy of autoreactive B cells serve to shape the peripheral B-cell repertoire. Nevertheless, dysregulation of B cell development and autoantibody production is a characteristic of most autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus, and type 1 diabetes, but also immunodeficiencies such as CVID, Wiskott–Aldrich Syndrome, and X-linked agammaglobulinemia (Park et al., 2005; Cuss et al., 2006; Westerberg et al., 2008). In ADA deficiency, some of the observed immune dysregulation were hypothesized to be associated with a more restricted B-cell repertoire due to abnormalities in central B-cell generation or to a dysregulated expansion of these cells in the periphery.

Autoantibodies appear in the serum many years before the onset of clinical disease suggesting an early break in B-cell tolerance (Wardemann et al., 2003). Some of B-cell mediated autoimmune diseases, such as myasthenia gravis (MG), idiopathic autoimmune thrombocytopenic purpura (AITP), and Graves' disease are characterized by auto-Abs production that destroy target tissues (Barsalou et al., 2011; Cunningham-Rundles, 2011). A remarkably high proportion of autoantibodies associated with systemic autoimmune diseases binds DNA, RNA, or macromolecular complexes that contain DNA or RNA. It has been hypothesized, that under certain circumstances these intracellular autoantigens become visible to the immune system when they accumulate during apoptosis. In fact the impaired clearance of apoptotic cell debris and dsDNA by macrophages might induce TLR signaling and differentiation of autoreactive B cells (Gaipl et al., 2006). Response to nucleic acid-containing immunecomplexes relies on the coengagement of endosomal members of the TLR family, TLR9 and TLR7 (Marshak-Rothstein, 2006). Therefore, self-antigens that can effectively engage both the BCR and either TLR7 or TLR9 might stimulate autoreactive B cells that are normally quiescent, through inherent adjuvant activity and trigger the development of systemic autoimmune disease (Marshak-Rothstein, 2006). In ADA deficiency, the metabolic basis underlying immune cell deficiency is the cytotoxic effect impact of the ADA substrates deoxyadenosine and dATP, leading to apoptosis of lymphocytes. It is therefore conceivable that developing B lymphocytes in affected lymphoid organs encounter massive amounts of nucleic acid. Nucleic acid-sensing TLRs might therefore represent Achilles' heel in susceptible ADA-deficient patients by which relative tolerance for nucleic acid-containing antigens is breached and autoimmunity occurs (Kono et al., 2009).

NEW INSIGHTS INTO IMMUNE CELL DYSFUNCTION AND ONSET OF AUTOIMMUNITY IN ADA DEFICIENCY

Recent in-depth studies have revealed specific defects in ADA deficiency that may contribute to the onset of autoimmunity in these patients. Herein we discuss alterations in the adenosinergic machinery of ADA-deficient regulatory T cells and in B-cell tolerance in the absence of functional ADA.

DEFECTIVE REGULATORY T-CELL FUNCTION IN ADA DEFICIENCY

Although autoimmune manifestations are frequent findings in ADA-deficient patients with milder forms or in patients under PEG-ADA, mechanisms causing the loss of peripheral tolerance and onset of autoimmunity have remained elusive. CD4+CD25+FoxP3+ Tregs actively suppress pathological and physiological immune responses in order to maintain peripheral immune self-tolerance and prevent autoimmunity (Sakaguchi et al., 2008; Sitkovsky et al., 2008). Extracellular adenosine produced by Tregs has been described as one of the mechanisms mediating their suppressive activity (**Figure 5A**). Concordant expression of the ectoenzymes CD39 and CD73 has been reported both for murine and human Tregs (Borsellino et al., 2007; Deaglio et al., 2007; Mandapathil et al., 2010). The CD39 ectoenzyme produces AMP from ATP or ADP, which is subsequently converted into extracellular adenosine by the CD73 ectoenzyme (Hasko et al., 2008). Treg function requires the coordinated expression of the Adora2a on activated T effector cells to enable adenosine-mediated immunosuppression (Sitkovsky et al., 2008). Moreover, Tregs have been shown to express low levels of ADA, whereas T effector cells are enriched in ADA but express low levels of CD39 and CD73 (Mandapathil et al., 2010; Sauer et al., 2012a). This molecular profile of Tregs (CD39+CD73+ADA*low*) has functional importance, as it not only confers Tregs the capability to produce extracellular adenosine but also to sustain relatively high concentrations due to low ADA expression (Mandapathil et al., 2010).

Figure 5B summarizes recently described defects and functional alterations of the adenosinergic pathway in Tregs from ADA-deficient mice and patients (Sauer et al., 2012a). ADA−/− Tregs showed significantly higher expression of CD39, while expressing significantly less CD73. ADA−/− Tregs are sensitive to extracellular adenosine concentrations and the expression of CD73 is regulated by this metabolite. With adenosine accumulating in ADA−/− mice, possibly to avoid a further increase of extracellular concentrations, CD73 is reduced and ADA−/− Tregs display a

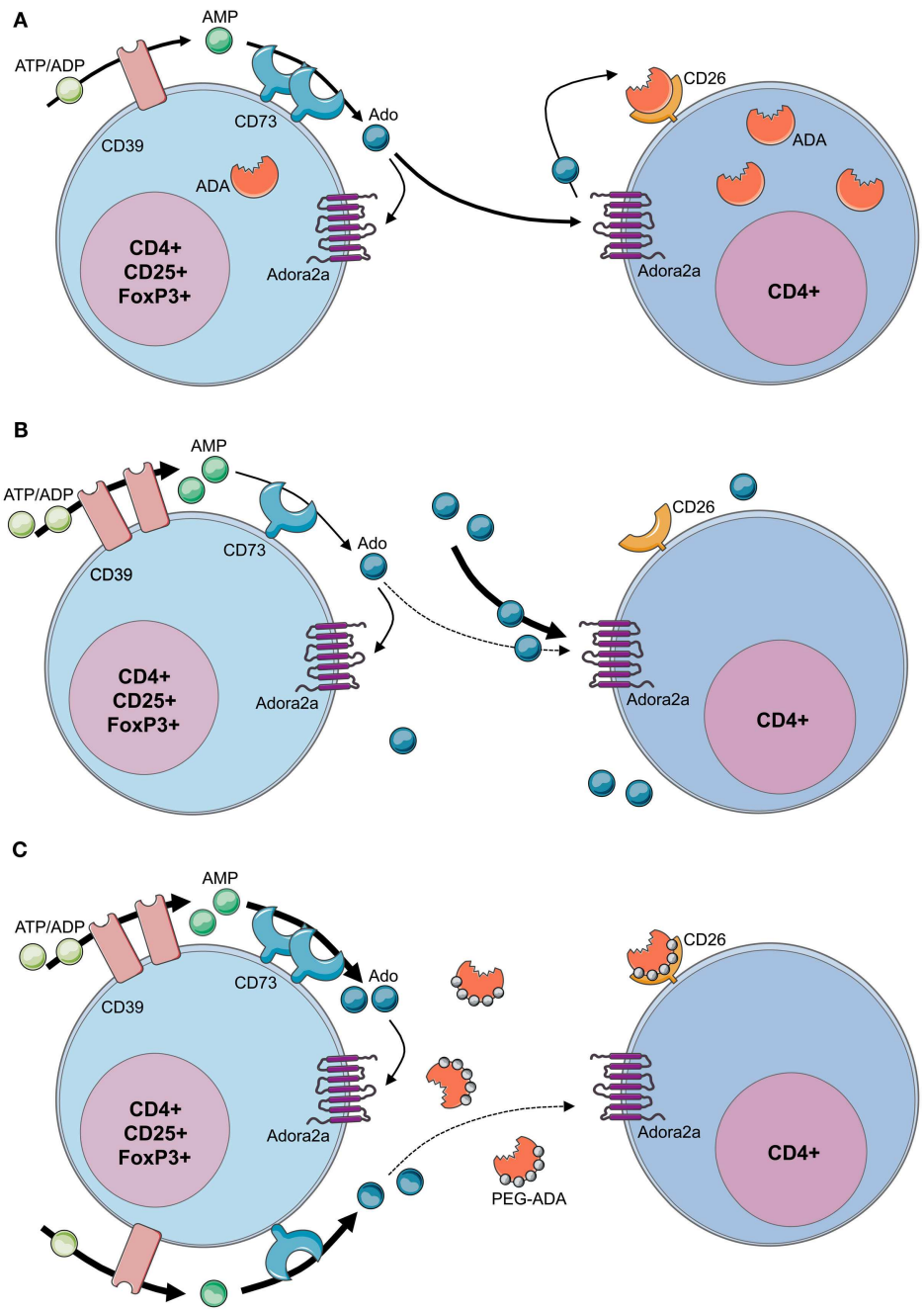

FIGURE 5 | Loss of regulatory T-cell function in ADA-SCID. (A) By concomitant expression of CD39 and CD73, Tregs have the enzymatic machinery to generate and maintain high levels of extracellular adenosine. Contrarily to T effector cells, Tregs express low levels of ADA and CD26. Extracellular ATP or ADP is converted by the ectonucleotidase CD39 into AMP, which is further converted into adenosine by the CD73 ectoenzyme. The produced adenosine binds to the Adora2A receptor expressed on activated effector T cells, which are enriched in ADA and the surface-bound glycoprotein CD26. The coordinated expression of CD39 and CD73 on Tregs and Adora2a on T effector cells enables adenosine-mediated immunosuppression. **(B)** In the absence of ADA, deficient Tregs express high levels of CD39, increasing their capacity for ATP hydrolysis, but reduced levels of CD73 on their surface. Although both CD39 and CD73 are rate limiting for extracellular adenosine generation, CD73 is the last component of the ectoenzymatic chain. With accumulating adenosine levels, possibly to avoid a further increase of extracellular concentrations, CD73 is reduced and ADA−/− Tregs display a decreased suppressive activity toward T effector cells. Nevertheless no apparent autoimmune manifestations can be observed at onset, likely due to the severely reduced effector T- and B-cell populations. Moreover, the accumulating extracellular adenosine is likely to maintain an anti-inflammatory environment in these mice. **(C)** After PEG-ADA treatment, the adenosinergic machinery of CD39 and CD73 are upregulated, indicating an increased requirement for ATP hydrolysis and enhanced adenosine production. Despite the initial rescue of suppressive activity by upregulation of CD73 for elevated adenosine production, long-term PEG-ADA treatment interferes with Treg function by augmenting adenosine turnover. PEG-ADA present in the extracellular space eliminates adenosine produced by the ectoenzymatic chain and hinders adenosine-mediated suppression by interfering between adenosine and Adora2a expressed on T effector cells.

decreased suppressive activity toward T effector cells. The underlying mechanism accounting for increased CD39 expression in ADA−/− Tregs remains to be elucidated. However, intracellular cAMP levels, which are elevated in the absence of ADA, have been reported to increase CD39 expression (Liao et al., 2010).

In order to dissect the cellular mechanisms leading to loss of peripheral tolerance, ADA−/− mice were studied after treatment with PEG-ADA, HSC-GT, and BMT. Although short-term PEG-ADA treatment initially rescued Treg-mediated suppression in comparison to untreated ADA−/− mice, their functionality became exhausted by long-term PEG-ADA treatment. Tregs from PEG-ADA treated animals maintained increased expression of CD39 and upregulated CD73 expression in comparison to age-matched wildtype controls. Consistently, CD39 activity measured by ATP consumption and AMP formation, as well as adenosine production by CD73 were significantly increased in comparison with wildtype Tregs. These results were confirmed in a cohort of patients including 7 PEG-ADA treated and 11 retroviral HSC-GT treated patients. The percentage of CD4+CD25+FOXP3+CD127−/low Tregs was significantly reduced in PEG-ADA treated patients and their expression of CD39 and CD73 ectonucleotidase were significantly increased. Unlike Tregs from HSC-GT treated patients and HD, Tregs isolated from PEG-ADA treated patients were unable to suppress the proliferation of effector cells (Sauer et al., 2012a).

The obtained results revealed an elevated adenosine catabolism in the presence of PEG-ADA, characterized by alterations in the adenosinergic machinery producing high levels of adenosine and a significantly increased turnover by the enzymatic activity of PEG-ADA. Upregulation of CD73 in treated ADA−/− mice and patients can therefore be interpreted as a compensatory mechanism representing a higher requirement for ATP/ADP to adenosine conversion in the presence of extracellular PEG-ADA. Despite the initial rescue of suppressive activity by upregulation of CD73 for elevated adenosine production, long-term PEG-ADA treatment interfered with Treg function by augmenting adenosine turnover. These findings fit the hypothesis that PEG-ADA present in the extracellular space eliminates adenosine produced by this ectoenzymatic chain and hinders adenosine-mediated suppression by interfering between adenosine and Adora2a expressed on T effector cells (Sauer et al., 2012a; **Figure 5C**).

DEFECTS IN B-CELL TOLERANCE IN ADA-SCID

Although PEG-ADA induces metabolic detoxification, BMT and HSC-GT provide superior restoration of purine metabolism and immune functions. However, it had remained unclear how patient's B cells contribute to autoimmune complications and if B-cell tolerance is established properly in ADA-deficient patients before and after treatment.

Random V(D)J recombination produces large numbers of antibodies displaying self-reactive specificities and during normal B-cell development the majority of these antibodies are removed at two distinct checkpoints in the bone marrow and periphery (Wardemann et al., 2003). Large numbers of self-reactive antibodies are removed from the B cell repertoire during the immature B cell stage in the bone marrow, where BCR-mediated selection plays a crucial role in controlling B-cell survival based on excessively strong or weak BCR signals that identify autoreactive or functionally unfit B cells (Goodnow, 1996; Nemazee et al., 2000; Cancro, 2009; **Figure 6**). Alterations of BCR signaling thresholds result in a defective central B-cell tolerance checkpoint and interfere with the removal of developing autoreactive B cells in humans (Ng et al., 2004; Menard et al., 2011). In addition to their BCRs, B cells also express TLRs that were originally described to bind microbial components but that are also able to recognize self-antigens (Marshak-Rothstein, 2006) and are involved in the removal of developing anti-nuclear antibody (ANA)-expressing B cells (Isnardi et al., 2008). Both BCR- and TLR-mediated B-cell responses have been reported to be modulated by adenosine receptor signaling and intracellular cyclic adenosine monophospate (cAMP), which are increased in ADA deficiency (Apasov et al., 2001; Hershfield, 2005; Minguet et al., 2005; Power Coombs et al., 2011).

B-cell tolerance checkpoints in ADA-SCID patients were assessed by cloning antibodies expressed by single B cells before and after successful HSC-GT (Sauer et al., 2012b). New emigrant/transitional and mature naïve B cells from ADA-deficient patients before HSC-GT contained high frequencies of autoreactive and ANA-expressing clones compared to healthy donors, revealing defective central and peripheral B-cell tolerance checkpoints in the absence of functional ADA.

The receptor candidates for the removal of ANA-expressing clones are nucleic acid-sensing endosomal members of the TLR family, TLR7 and TLR9 (Marshak-Rothstein, 2006), thereby suggesting that ADA impinges not only on BCR but more importantly TLR signaling. A similar mechanism has also been hypothesized to contribute to B-cell dysfunctions, defective B-cell proliferation, and activation observed in ADA-deficient mice (Aldrich et al., 2003; Hershfield, 2005). The accumulating adenosine blocks NF-κB activation in murine B cells stimulated through BCRs or TLR4 by LPS (Minguet et al., 2005; Power Coombs et al., 2011). In line with this hypothesis, we found that stimulation through TLR7 and TLR9 were significantly dependent on proper ADA enzymatic activity and adenosine receptor engagement, further underlining the inability of these receptors to function in the absence of functional ADA (**Figure 6**).

Strikingly, ADA-deficient patients treated with HSC-GT displayed quasi-normal early B-cell tolerance checkpoints as evidenced by restored efficient removal of developing autoreactive and anti-nuclear B cells. Hence, ADA plays an essential role in the establishment of early B-cell tolerance and the removal of developing autoreactive B cells in humans (Luning Prak, 2012; Sauer et al., 2012b).

CONCLUDING REMARKS ON THE OCCURRENCE OF AUTOIMMUNITY AFTER ADA-SCID TREATMENT

In summary, the available literature provides supporting evidence for a predisposition to autoimmunity in ADA deficiency. Alterations in both central and peripheral tolerance in T- and B-cells have been described to contribute to the pathogenesis of autoimmunity. Moreover, it is becoming increasingly clear that tolerance mechanisms and immune responses are specifically altered by the lack of ADA and the accumulation of its substrates.

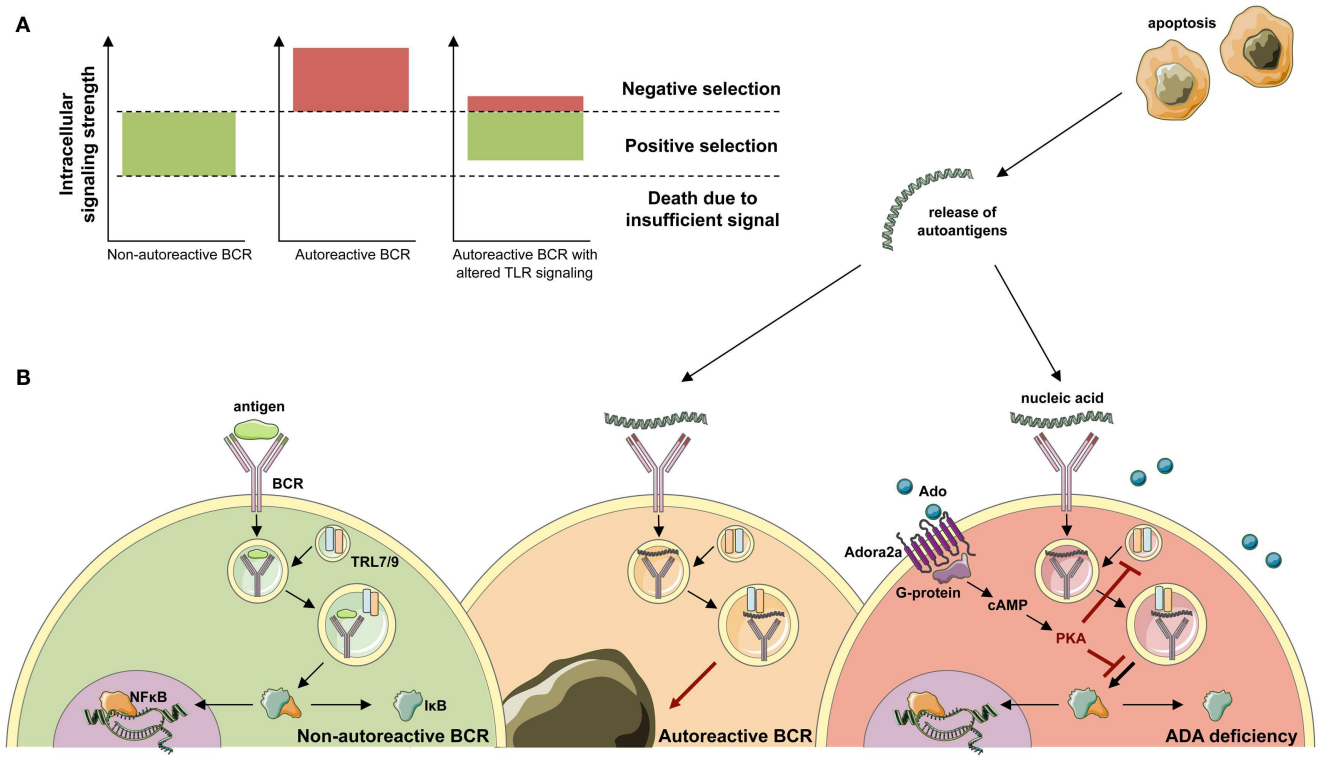

FIGURE 6 | Development of ANA-expressing B-cell clones in ADA deficiency. (A) During B-cell development in the bone marrow, BCR signals provide a cell-intrinsic measure for negative or positive selection. By excessively weak or strong BCR signals, two thresholds identify functionally unfit or autoreactive B-cell clones. B cells that fail to rearrange and signal through their BCR die of neglect. Negative selection against autoreactive BCR specificities occurs following high avidity BCR interactions with antigen. While positive selection of B-cells requires persistent intermediate BCR signaling in both developing and mature B-cells. **(B)** Upon antigen-binding the BCR is internalized and transported to the cytoplasmic compartments containing Toll-like receptor 9 (TLR9) or TLR7. Physiological engagement of immunoreceptors leads to the phosphorylation and proteasomal degradation of IκB, thereby releasing NF-κB into the cytoplasm. Subsequently, NF-κB translocates to the nucleus and initiates the transcription of NF-κB target genes required for immune cell development and function. During the bone marrow differentiation of B cells, a major source of autoantigen is developing lymphocytes, which undergo apoptosis. Autoantigens are released in the extracellular space and form immune complexes. Binding of nucleic acid-containing immune complexes to an autoreactive BCR produces a strong activation signal through simultaneous activation of TLRs and leads to the depletion or receptor editing of the B cell. It can be hypothesized that in ADA deficiency this negative selection is dampened by adenosine present in the extracellular place and engagement of the Adora2a. Activation of Adora2a elevates intracellular cAMP through activation of adenylyl cyclase. In turn, cAMP activates PKA that blocks the BCR-induced phosphorylation of IκB to inhibit immunoreceptor-mediated NF-κB activation in the cytoplasm. The strong depletion signal coming from BCR and TLR coengagement is thereby lowered and ADA-deficient B cells carrying an autoreactive BCR egress into the periphery.

Particularly, the impact of accumulating adenosine as anti-inflammatory mediator has to be underlined in ADA deficiency. The ligation of Adora2a receptors leads to an increase in cAMP levels, which in cooperation with PKA induces immunosuppression, attenuation of proximal signaling events after TCR and BCR triggering, and inhibition of downstream effector functions (Skalhegg et al., 1992; Huang et al., 1997; Lappas et al., 2005; Cassani et al., 2008; Sauer et al., 2012b). It can further be hypothesized that dampening of TCR- and BCR-downstream signaling interferes with the depletion signals during negative selection in central tolerance, thereby allowing the survival of autoreactive cells in ADA deficiency. In addition to its effects on T- and B-cells, adenosine is an important regulator, physiologically involved in inhibiting a variety of activated immune cells and in protecting tissues from acute inflammatory damage (Panther et al., 2003; Sitkovsky et al., 2004). Indeed we showed that Tregs require a balanced adenosine metabolism to exert their suppressive activity, since excessively high adenosine concentrations, or excessive conversion of extracellularly produced adenosine by PEG-ADA interferes with their suppressive function (Luning Prak, 2012; Sauer et al., 2012a).

The precise role of PEG-ADA alongside other treatment options is still undetermined, but it certainly allows rapid detoxification and stabilization of patients awaiting more definitive treatment (Booth and Gaspar, 2009). With a progressive loss of lymphocyte functions, the occurrence of neutralizing anti-ADA antibodies and autoimmune manifestations, long-term immunological reconstitution in PEG-ADA patients is often incomplete. It has been hypothesized that partial ADA correction resulting in low enzymatic activity may mimic late-onset patients, which typically display a higher prevalence of autoimmune manifestations (Ochs et al., 1992; Ozsahin et al., 1997; Luning Prak, 2012). Indeed, recent data underlined the importance of intracellular ADA expression

and superior efficacy of gene therapy over PEG-ADA treatment for the development of functional T- and B-cell tolerance, including Tregs (Sauer et al., 2012a,b).

Both BMT and HSC-GT provide efficient reconstitution of the immune system through endogenous enzymatic ADA activity. BMT from an HLA-identical sibling donor remains the treatment of choice, while transplants from alternative donors are associated with high morbidity and mortality. The occurrence of mixed chimerism in transplanted patients with other primary immunodeficiencies is associated with a higher incidence of autoimmune manifestations (Moratto et al., 2011) and might well play a role also in ADA deficiency (Cancrini et al., 2010). Moreover, transplantation-induced lymphopenia is a possible cause for the homeostatic expansion of autoreactive T- and/or B-cells with subsequent loss of self-tolerance (Daikeler and Tyndall, 2007). Phenomena of immune dysregulation as occurring in the context of pre-transplant conditioning and BMT might further trigger the onset of autoimmunity (Etzioni, 2003).

In accordance with the current guidelines of the European group for Blood and Marrow Transplantation (EBMT) and given the long-term experience in gene therapy (Aiuti et al., 2009), this treatment can now be considered for all ADA-SCID patients lacking an HLA-identical sibling donor (Gaspar et al., 2009). After HSC-GT, high levels (50–90% on average) of gene correction were detected in T- and B- and NK-cells (Aiuti et al., 2009), leading to an efficient systemic detoxification and recovery of immune cell functions. However, as suggested by the cloning of single B-cell receptors, non-gene corrected cells may carry autoreactive specificities (Sauer et al., 2012b). It can be hypothesized that the co-existence of non-corrected autoreactive T- or B-cells and the restored functional T cell help could allow the development of autoimmune manifestations in ADA-SCID patients after HSC-GT (Aiuti et al., 2009). Modification of preparatory regimen or increased gene transfer efficiency by more robust approaches such as lentiviral vectors may further improve HSC-GT outcome for ADA deficiency (Mortellaro et al., 2006).

Adenosine deaminase-SCID remains a challenging condition to treat (Gaspar et al., 2009). With large-scale outcome studies still lacking, the choice between lifelong PEG-ADA, unrelated BMT and HSC-GT is currently based on the risk/benefit ratio, the availability, and costs of the three different treatment options (Gaspar et al., 2009). Due to the rarity of the disease and the small cohort numbers, accurate survey and long-term follow-up will be essential to determine the outcome following different treatments and their efficacy in restoring immune tolerance.

ACKNOWLEDGMENTS

Supported by grants from the Italian Telethon Foundation: TIGET, core grant A1; the European Commission: Advanced Cell-based Therapies for the treatment of Primary ImmunoDeficiency (Cell-PID), HEALTH F5-2010-261387; and the Ministero della Salute: Ricerca Finalizzata, 005/RF-2009-1485896. **Figures 2–6** were produced using Servier Medical Art.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 May 2012; accepted: 02 August 2012; published online: 27 August 2012.

Citation: Sauer AV, Brigida I, Carriglio N and Aiuti A (2012) Autoimmune dysregulation and purine metabolism in adenosine deaminase deficiency. Front. Immun. ***3****:265. doi: 10.3389/fimmu.2012.00265*

This article was submitted to Frontiers in Primary Immunodeficiencies, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 14 August 2012
doi: 10.3389/fimmu.2012.00234

The STAT5b pathway defect and autoimmunity

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The signal transducer and activator of transcription (STAT) 5b is a universal transcription factor that plays key biological roles in allergic diseases, immunodeficiencies, autoimmunities, cancers, hematological diseases, growth disorders, and lung diseases. The identification of distinct pathological manifestations of STAT5b deficiency in humans has highlighted the critical role of the STAT5b pathway. Proper gene transcription at *IL-2Rα*, *FOXP3*, *Bcl-2*, and growth hormone (GH) associated loci are thought to be associated with normal STAT5b transcriptional activity. These genes are thought to play important roles in allergy/autoimmunity, immunodeficiency, cancer/anemia, and growth, respectively. The *STAT5A* and *STAT5B* genes are collocated on 17q11. Although these two monomeric proteins exhibit peptide sequence similarities of >90%, it is known through observations of STAT5b deficient subjects that STAT5a and STAT5b are not fully redundant in humans. Patients with STAT5b deficiency have decreased numbers of regulatory $CD4^+CD25^{high}$ T cell (Treg) despite their STAT5a levels being normal. Prior studies on STAT5b deficient subjects have revealed immunological aberrations associated with the following disease phenotype: modest lymphopenia and decreased populations of Treg, γ-δ T cells, and natural killer (NK) cells. Most subjects with STAT5b deficiency show severe eczema, and autoimmune disease (juvenile idiopathic arthritis, autoimmune thyroiditis, idiopathic thrombocytic purpura) which are thought to be associated with Treg dysfunction. We will review the likely pathophysiological mechanisms associated with STAT5b deficiency.

Keywords: allergy, autoimmunity, IL-2, immunodeficiency, STAT5b, CD25, Foxp3, Bcl-2

INTRODUCTION

The signal transducer and activator of transcription (STAT) 5b is a universal transcription factor that plays key biological roles in allergic disease, immunodeficiencies, autoimmunities, cancers, hematological disease, growth disorders, and lung disease (Buggins and Pepper, 2010; Nadeau et al., 2011).

There are several differences between human and mouse in the roles of STAT5b (Nadeau et al., 2011). The identification of STAT5b deficiency in humans, and the distinct and destructive pathology associated with this deficiency has highlighted the critical role the STAT5b pathway. Research on the immunologic function of STAT5b has demonstrated its importance for the *in vivo* accumulation of regulatory $CD4^+CD25^{high}$ T cells (Treg) with immunoregulatory function (Cohen et al., 2006; Nadeau et al., 2011). The specific role that STAT5b plays in the pathogenesis of the aforementioned diseases has led to suggestions that the transcription factor might have potential as a novel diagnostic and/or therapeutic target in some disease settings.

In this review, we summarize recent advances in our understanding of the STAT5b pathway in human mainly as well as the autoimmune manifestations induced by the defects within it.

THE STAT5b PATHWAY

STAT5b GENE AND PROTEIN, AND NON-REDUNDANCY BETWEEN STAT5a AND STAT5b

The *STAT5B* gene is collocated on 17q11.2 approximately 12 kb apart from *STAT5A* (**Figure 1**). Both genes are regulated by a Sp-1 cis-element (Crispi et al., 2004).

Although STAT5a and STAT5b show peptide sequence similarities of >90%, they differ by six amino acid in the DNA binding domain and 20 amino acids in their carboxy termini (Boucheron et al., 1998; Grimley et al., 1999; Soldaini et al., 2000; Wei et al., 2008). Additional reports of a common disease phenotype specifically associated with STAT5b deficiency in humans (but no such phenotype associated with STAT5a deficiency) indicates that, at least in humans, the roles of STAT5a and STAT5b are not fully redundant (Nadeau et al., 2011).

Structural dissimilarities between the STAT5a and the STAT5b on transactivation domains or subtle differences in the DNA binding affinities of STAT5 dimer pairs could influence gene regulation, but cell-dependent asymmetries in the availability of phosphorylated STAT5a or STAT5b could also another factor. Signal attenuation by phosphatase action or classic feedback inhibition, or truncated forms of STAT5b lacking in transactivation capacity, may compete upstream for activation and diminish access of full length molecules to DNA binding sites (Grimley et al., 1999). Thus, both STAT5 proteins could bind to the same targets, and any differences between STAT5a and STAT5b may arise from differential expression or difference in kinetics of DNA binding (Grimley et al., 1999).

UPSTREAM OF STAT5b: CYTOKINES AND THEIR RECEPTORS

Signal transducer and activator of transcription 5b is a common downstream effector of the IL-2, -4, -7, -9, -13, -15, -21, growth hormone (GH; Liu et al., 1997), erythropoietin, thrombopoietin, and granulocyte colony-stimulating factor signaling molecules

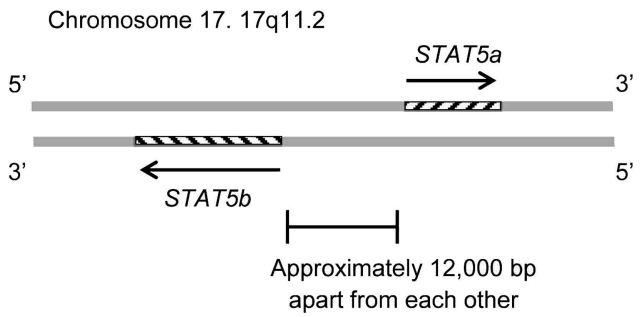

FIGURE 1 | The STAT5B gene is collocated on 17q11.2 approximately 12 kb apart from the STAT5A gene. The STAT5B gene is on the negative strand, and the STAT5a gene is on the positive strand. The genomic size of STAT5b is 58,700–77,229. The genomic size of STAT5a is approximately 24,000.

(Nadeau et al., 2011). Each cytokine has associated receptors, and each receptor has associated Janus kinases (JAK). For example, the IL-2 receptor is composed of an α chain (CD25), β chain (CD122), and γ chain (CD132; Lin and Leonard, 2000). The β chain is associated with JAK1 and JAK3 (Zhu et al., 1998) and the γ chain is associated with JAK3 (**Figure 2**; Russell et al., 1995). The growth hormone receptor (GHR) is associated with JAK2 (Hwa et al., 2011).

The CD25 plays an important role as an integral component of the high affinity IL-2 receptor. Its ligand, IL-2, is a cytokine known for the role it plays in lymphocytic function, especially with relation to T cell biology. There are two functional receptors for IL-2: one is a heterodimeric complex formed by the β and γ chains, while the other is a trimeric membrane-spanning complex composed of the α, β, and γ subunits. The latter receptor has a higher affinity for IL-2 than the former (Lin and Leonard, 2000). Additionally, defects in STAT5b expression and function have been shown to result in reduced expression of IL-2Rα, thereby potentially limiting cellular response to IL-2 signaling (Cohen et al., 2006).

The engagement between cytokines and their cell surface receptors results in subsequent activation of receptor-associated JAK tyrosine kinase activity. Activated JAKs phosphorylate specific tyrosine resides in the cytoplasmic domain of their associated receptor, and these newly phosphorylated residues serve as docking sites for STAT proteins (**Figure 2**; Grimley et al., 1999).

PHOSPHORYLATION OF STAT5b BY JAKs (MAINLY JAK1 AND 3)

Intracellular signal transduction pathways are essential for transforming extracellular cytokine signaling into appropriate cellular responses. The phosphorylation of STAT molecules is a key component in the JAK/STAT signal transduction pathway (Xu and Qu, 2008).

Cytokine engagement of membrane-associated receptors brings receptor subunits into proximal relationships necessary for JAK autophosphorylation (**Figure 2**). Cytoplasmic STAT monomers are subsequently able to bind the phosphotyrosine residues on engaged cytokine receptors through the highly conserved SH2 domain located on all proteins of the STAT family (**Figure 3**).

As a result of this docking, JAK and STAT molecules are brought into close enough proximity to allow for JAK phosphorylation, and therefore activation, of STAT molecules. In the case of STAT5, phosphorylated STAT5a and/or STAT5b then homo- or heterodimerize (sometime tetramaerization; John et al., 1999; Soldaini et al., 2000; Mandal et al., 2011) by each SH2 domain, leave the receptor, and translocate to the nucleus where they act as a transcriptional activator for each target gene (Levy and Darnell, 2002).

DOWNSTREAM OF THE STAT5b PATHWAY

Signal transducer and activator of transcription 5b dimers translocate into the nucleus and bind to specific regions thought to be associated with transcription of *FOXP3*, *CD25*, *Bcl-2*, *IGF-1* (Nadeau et al., 2011). Reports indicate that STAT5b may preferentially interact with different DNA binding sites depending on the cell type considered.

Fork-head box P3 (FOXP3): a key transcription factor essential for Treg cell development and function

The transcription factor FOXP3 is critical for the thymic development of Tregs (Sakaguchi et al., 2008). In mice, $CD4^+CD25^+$ peripheral T cells and $CD4^+CD25^+CD8^-$ thymocytes express Foxp3 and are considered to be immunoregulatory, whereas other thymocytes/T cells, either in a resting or activated state, do not (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Sakaguchi et al., 2008).

Studies investigating the effects of FOXP3 suppression report complications associated with Treg dysfunction to be a main pathological consequence. Mutations of the *FOXP3* gene were found to be the cause of an IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is characterized by autoimmune disease in multiple endocrine organs (as in type I diabetes and thyroiditis), inflammatory bowel disease, and severe allergy (Chatila et al., 2000; Bennett et al., 2001; Wildin et al., 2001). Deletion or dysfunction of FOXP3 causes impaired function and/or homeostasis of Tregs, and has been implicated in the development of several common autoimmune and inflammatory diseases (Campbell and Koch, 2011).

The essential role of CD25 in Treg development and function

High expression of CD25 is considered to be a marker of Tregs (Sakaguchi et al., 1995) and studies have elaborated on this concept, demonstrating that the IL-2Rα serves not only as a marker for natural Treg, but also, as a protein essential for its development and function (Sakaguchi et al., 2008). The importance of CD25 in the development of a normal immune response is emphasized by the finding that a truncation mutant of CD25 results in an immunodeficiency in humans characterized by an increased susceptibility to viral, bacterial, and fungal infection (Sharfe et al., 1997). In addition, gene targeting analysis also reveals that CD25 deficient mice exhibit autoimmunity (Willerford et al., 1995).

While CD25 contributes to IL-2 binding affinity and not to the recruitment of signaling molecules (Lin and Leonard, 2000) its role as a component of the high affinity IL-2 receptor makes it indispensable for the activation of cell signaling pathways associated with IL-2 signal transduction (Sakaguchi et al., 2008).

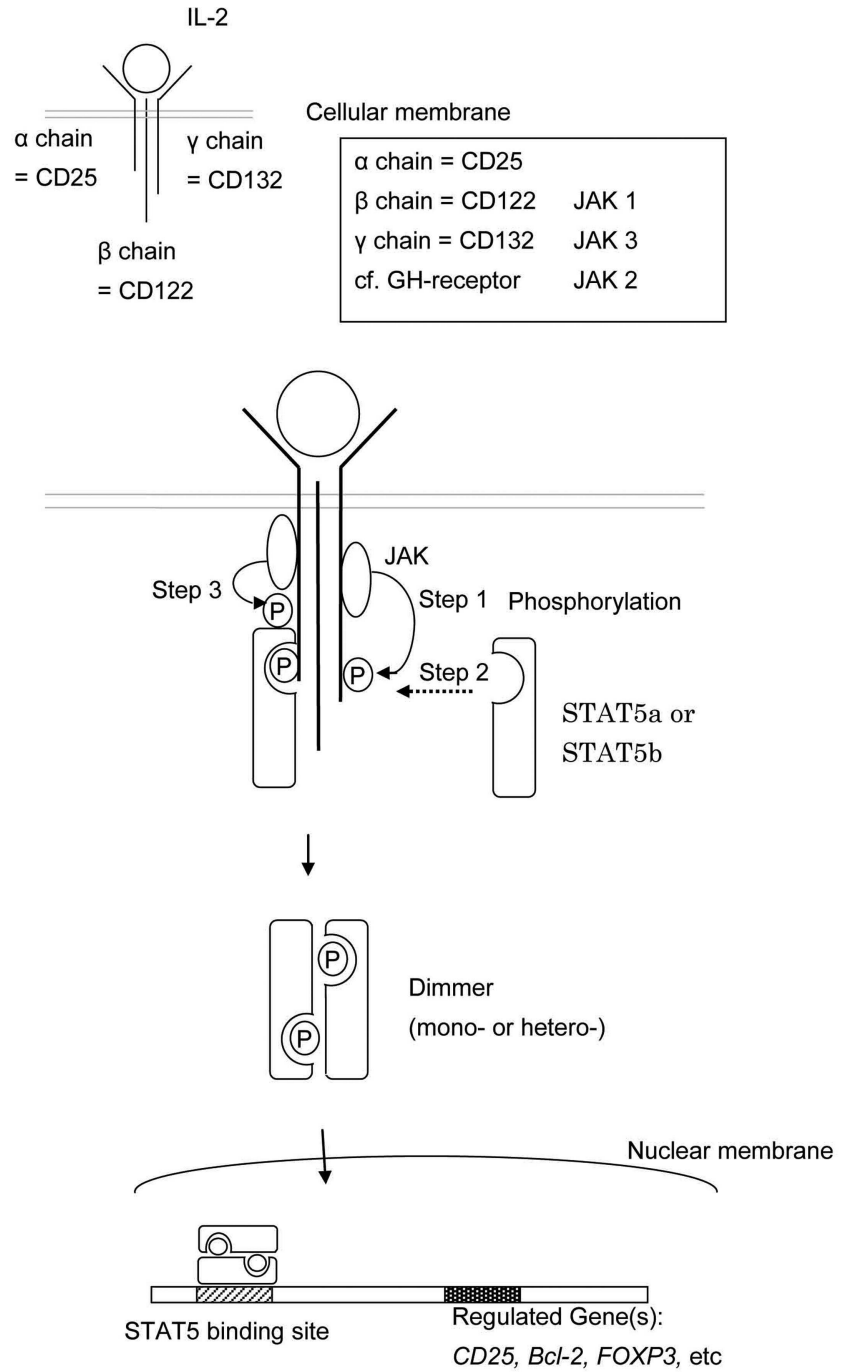

FIGURE 2 | This shows the schema of STAT5a and/or STAT5b activation. The engagement between a cytokine and its cell surface receptor results in subsequent activation of receptor-associated JAK. Activated JAK phosphorylates specific tyrosine resides in the cytoplasmic domain of the receptor which in turn serves as the docking sites for STAT5a and/or STAT5b. STAT5a and/or STAT5b are recruited to the phosphorylated receptor and subsequently phosphorylated by JAKs. The phosphorylated STAT5a and/or STAT5b dimerize, leave the receptor, and translocate to the nucleus.

Bcl-2 is an apoptotic inhibitor protein

Bcl-2 is an apoptosis inhibitor protein. Most cell death in vertebrates occurs via the mitochondrial pathway of apoptosis, in which Bcl-2 and other anti-apoptotic proteins (Bcl-xL, Bcl-w, Mcl-1, and Bfl-1/A1) are key effectors (Llambi and Green, 2011). Aberrant regulation of Bcl-2 has been reported to cause or correlate with autoimmunity or cancer, particularly leukemia (Buggins and Pepper, 2010; Tischner et al., 2010). Deletion of self-reactive immune cells occurs through this apoptotic pathway and is necessary for the maintenance of immune tolerance (Tischner et al., 2010).

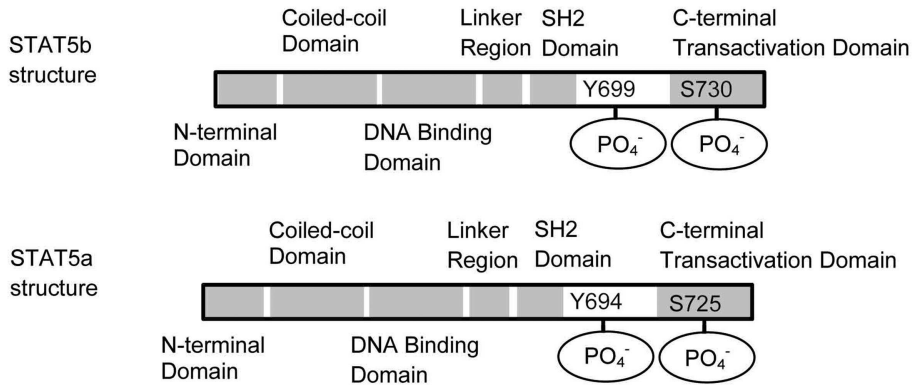

FIGURE 3 | Schematic structures of STAT5a and STAT5b. STAT5a and STAT5b differ in the C terminal domain. The dimerization occurs through the interaction between the SH2 domains.

Overexpression of Bcl-2 has been noted in patients with systemic lupus erythematosus (Tischner et al., 2010). In malignant diseases, decreased rate of apoptotic cell death is also found to be responsible in the proliferative process (Ulukaya et al., 2011).

Insulin-like growth factor-I and Insulin-like growth factor binding protein-3 play an important role in fat metabolism and skeletal development

Insulin-like growth factor-I promotes skeletal development and fat metabolism, and insulin-like growth factor binding protein-3 (IGFBP-3) acts as a negative regulator for IGF-I signaling (Kawai and Rosen, 2010).

The activation of IGF-I is initiated by the interaction of circulating GH with the GHR. The cytoplasmic domain of GHR associates preferentially with JAK2. Activation of JAK2 by GHR engagement leads to the activation of STAT5b (Hwa et al., 2011).

In humans, serum IGF-I concentrations have a positive correlation with skeletal mass (Langlois et al., 1998). A report on the disease characteristics of STAT5b deficiency in humans highlights low serum IGF-1 as one defining clinical feature of the disease (Hwa et al., 2004; Nadeau et al., 2011). STAT5b deficient patients also exhibit stunted growth and poor response to GH therapy (Nadeau et al., 2011).

IGF-I was also reported as a critical factor for adipogenesis (Kawai and Rosen, 2010). The lack of this factor results in a defect in adipose tissue formation by mitogen-activated protein kinase deactivation in conjunction with GH (Boney et al., 2000; Hwa et al., 2011).

IGFBP-3 suppresses adipogenesis independent of IGF-I binding (Chan et al., 2009) and reduces bone mineral density (Kawai and Rosen, 2010).

HUMAN STAT5b PATHWAY DEFECT AND AUTOIMMUNITY

Human STAT5b deficiency is a recently identified, rare autosomal recessive disease that involves both severe GH-resistant growth failure and severe primary immunodeficiency. It was first discovered in patients with dwarfism associated with normal levels of serum GH, but very low levels of IGF-I (Kofoed et al., 2003; Bernasconi et al., 2006; Chia et al., 2006). Affected individuals also exhibited recurrent infections, chronic diarrhea, eczema, and/or lymphocytic interstitial pneumonitis (Kofoed et al., 2003; Bernasconi et al., 2006; Chia et al., 2006). Immunophenotyping of these patients have revealed modest lymphopenia and decreased populations of Treg, γ-δ T cells, and natural killer (NK) cells (Bernasconi et al., 2006; Cohen et al., 2006). There are currently 10 published cases of STAT5b deficiency (**Table 1**; Nadeau et al., 2011). Ongoing research efforts aim to identify the molecular mechanisms of STAT5b in postnatal growth and immunity.

Previous cases

The first case of a STAT5b mutation was reported in 2003, in a 16-year-old female with severe growth retardation (−7.5 SD) and pulmonary complications (Kofoed et al., 2003). The reported missense mutation (p.A630P) disrupted the core of anti-parallel β-sheets that enable phosphate-binding, causing aberrant folding (Chen et al., 1998) aggregation of mutant STAT5b protein, and loss of thermodynamic stability (Chia et al., 2006; Fang et al., 2006). The patient presented with early onset lymphocytic interstitial pneumonitis, chronic lung disease, hemorrhagic varicella, atopy, and autoimmune disease (Kofoed et al., 2003). At age 7, she developed lymphocytic interstitial pneumonia and after receiving potent immunosuppressive therapy, had two major infectious complications – severe varicella-zoster virus infection and *Pneumocystis jiroveci* pneumonia. Another biopsy at age 10 also indicated lymphoid interstitial pneumonia, and *P. carinii* was isolated from the tissue. Later studies revealed decreased numbers of Treg and reduced Treg suppressive function (Cohen et al., 2006).

In 2005, a second case of a STAT5b deficiency was identified in a 16-year-old Turkish female with severe growth failure, GHI, atopic dermatitis, pruritic skin lesions, primary idiopathic pulmonary fibrosis with diffuse lung involvement, and autoimmune disease, as well as bleeding diathesis caused by defective thrombocyte aggregation, preventing a potential lung biopsy (Hwa et al., 2005). Sequencing of the *STAT5b* gene revealed a novel homozygous frameshift mutation (c.1191insG) that led to protein termination (p.N398EfsX16) and consequent lack of immunodetectable STAT5b protein (Hwa et al., 2005).

Another case was identified in 2006 in a 16-year-old female with severe postnatal growth failure, GHI, and immunodeficiency

Table 1 | Demographics of published cases with STAT5b deficiency.

	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	Reference values
STAT5b Mutation	*A630P* −/−	*1191insG* −/−	*R152X* −/−	*R152X* −/−	*1103insC* −/−	*1680delG* −/−	*1680delG* −/−	*424_427del* −/−	*424_427del* −/−	*F646S* −/−	
Place of origin	Argentina	Turkey	Argentina	Argentina	Caribbean	Kuwait	Kuwait	Brazil	Brazil	Argentina	
Sex	F	F	F	F	M	F	F	M	M	F	
Height, SDS	−7.5	−7.8	−9.9	−5.3	−5.9	−5.8	−5.6	−5.6	−3	-5.90	
Age	16.5	16.4	15.3	12	31	2	4	6	2	18	
Onset of chronic pulmonary disease	7 years	7 years	1 year	6 months	No	6 years	6 years	Yes	Yes	No	
Skin pathology	Eczema	Eczema	Eczema	Eczema	Ichytosis	No	No	Atopic	Atopic	Seborrheic dermatitis	
Autoimmune manifestations	+Abs to BEC	ITP + Abs to platelets	No	AIT with + Abs to thymoglobulin	Ichytosis + Abs to platelets	SJIA	No	No	No	AIT	
Parental consanguinity	Yes	Yes	NA	Adopted	NA	Yes	Yes	ND	ND	Adopted	
Paternal height, SDS	−0.3	−0.9	−2.2	NA	−0.8	−1.28	−1.28	−1.6	−1.6	NA	
Mother height, SDS	−1.2	−0.6	−3.3	NA	−2.8	−0.6	−0.6	−1.3	−1.3	NA	
Birth weight/length (cm)	1400/ND	2350/49	2500/ND	1650/ND	3270/50	2400/ND	3600/ND	1650/39	2400/49	2250/44	
Puberty	Delayed	Delayed	Delayed	NA	Delayed	NA	NA	NA	NA	NA	
BIOCHEMISTRY											
GH basal (ng/mL)	9.4	14.2	6.6	1.8	0.13	17.7	5.7	1.7	1	1.7	
GH stimulated (ng/mL)	53.8	NA	NA	12.5	14.2	NA	NA	20.6	14	27.1	>6.0
IGF-I stimulated (ng/mL)	38	7.0	<10	0.8	14	<5	<5	34	<25	16	119–483
IGFBP-3 (ng/mL)	874	543	NA	500	180	700	800	520	750	840	210-740
ALS (acid labile subunit; mg/L)	2.9	1.2	NA	0.7	0.7	0.4	0.8	520	750	NA	
References for published case	Cohen et al. (2006), Kofoed et al. (2003)	Hwa et al. (2005)	Bernasconi et al. (2006)	Bernasconi et al. (2006)	Vidarsdottir et al. (2006)	Hwa et al. (2007)	Hwa et al. (2007)	Pugliese-Pires et al. (2010)	Pugliese-Pires et al. (2010)	Scaglia et al. (2012)	

ITP, idiopathic thrombocytopenic purpura; AIT, autoimmune thyroiditis; Abs, antibodies; BEC, bronchial epithelial cells; SJIA, systemic juvenile idiopathic arthritis; ND, not diagnosed; NA, not currently available; PC, personal communication. The data of #6 and #7 in adjacent columns represent data from siblings.

Heterozygote STAT5b-deficient subjects who are relatives of homozygote STAT5b-deficient subjects currently have not been identified to have any abnormal clinical phenotypes, although a specific detailed review of their clinical demographics and laboratory studies has not been done yet.

(Bernasconi et al., 2006). Pulmonary-function tests showed mixed, restrictive, and obstructive moderate ventilative insufficiency, but no lung biopsy was performed. Notably, this case was the first to identify a role for STAT5b not only in the human GH signaling cascade, but also in the cytokine-mediated immune response. The STAT5b deficient patient had moderate T cell lymphopenia, normal CD4/CD8 ratios, and very low numbers of NK cells and γ-δ T cells, and the T cells presented a chronically hyperactivated phenotype (Bernasconi et al., 2006).

Since 2012, five other mutations have been published on a total of seven additional subjects (**Table 1**). Lung pathology has been common among these patients (8 of 10), but of these remaining seven subjects, only a few have received lung biopsies. A STAT5b deficient male with the mutation 424_427del received a biopsy at 6 years of age that indicated severe lymphocytic interstitial pneumonitis. Considered together, these studies have firmly established a correlation between STAT5b deficiency and immune dysfunction, in addition to GHI and severe growth problems.

Clinical manifestations and diagnosis

Signal transducer and activator of transcription 5b deficiency should be considered in the differential diagnosis of a patient who has normal gestational growth and birth size but acquires significantly short stature and recurrent infections. This pattern of growth is typical of patients with GHI. Height may range from −3.0 to −9.9 SD in girls and boys, respectively (**Table 1**).

Regarding hormone evaluations, all described patients have had normal levels of GH at baseline, but after stimulation, GH concentrations were often elevated (**Table 1**). In contrast, serum IGF-I, IGFBP-3, and acid labile subunit concentrations were low, and even upon administration of GH, remained low. Elevated prolactin levels were also observed in patients with recorded concentrations.

Most patients have displayed evidence of immune dysfunction, including atopic disease, chronic lung disease, viral infections, and/or autoimmune diatheses. Often present in childhood, severe pulmonary disease is of particular concern, as it has affected 8 of the 10 known STAT5b deficient patients and two patients have died of respiratory failure. For all cases of lung pathology except for that of Patient #5, an axial chest CT scan has shown increased interstitial patterns and ground-glass appearance. These pulmonary lesions are T cell predominant, despite peripheral lymphopenia. In most cases, severe eczema, thrombocytopenic purpura, and/or autoimmune disease, such as juvenile idiopathic arthritis, were present in addition to severe lung disease. However, it should be noted that 1 of the 10 subjects to date has less severe immune dysfunction. Congenital ichthyosis was diagnosed at birth, and the patient had hemorrhagic varicella at 16 years of age but had no history of pulmonary of immunological problems (Vidarsdottir et al., 2006).

Previous immunological studies have established the importance of STAT5b proteins in the development, homeostasis, and proliferation of different lymphocyte populations. Immune repertoires of STAT5b deficient patients have shown moderate lymphopenia, with very low numbers of NK and T cells, as well as Treg dysfunction. Furthermore, B cell populations and immunoglobulin G levels in at least patient are normal to elevated, as consistent with autoimmune disease symptoms (Cohen et al., 2006).

Disease management

In order to improve clinical outcomes for patients with STAT5b deficiency, optimizing early diagnosis in these patients is critical. To date, overall management of STAT5b deficiency is still unclear. GH therapy is ineffective due to the patients' GHI. It is presumed that IGF-I therapy may be an effective treatment, unless the presence of chronic infection limits the growth response. However, to date, no clinical trials of IGF-I therapy have been performed in these patients.

Patients should be closely monitored for signs and symptoms of immunodeficiency. Infections such as severe varicella or recurrent pneumonias should be aggressively treated with appropriate antimicrobial therapies. Patients with autoimmune conditions, atopic diseases, or pulmonary fibrosis may also require antiproliferatives or immunosuppressants, such as steroids, to address overactive effector T cell responses. Because severe chronic lung disease in this patient population often leads to high morbidity and mortality, patients should be carefully monitored with pulmonary-function tests and physical examinations, which may improve treatment options to decrease the lung disease severity.

Although current management of STAT5b deficiency is primarily dictated by specific end-organ pathology, current research is addressing the possibility of enhancing STAT5b and/or STAT5a pathways (Zeiser et al., 2008; Strauss et al., 2009). Future therapy may be expected to prevent and reflect rationally based drug design to enhance certain drug targets in the STAT5b and/or STAT5a pathways.

CONCLUSION

In this review, we focused on the STAT5b pathway and the mechanisms by which defects in protein structure and or expression might result in autoimmunity. A better understanding of STAT5b and its distinct biological functions is necessary for the development of new diagnostic and therapeutic approaches for treating patients suffering from its deficiency.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 May 2012; paper pending published: 27 May 2012; accepted: 15 July 2012; published online: 14 August 2012.
*Citation: Kanai T, Jenks J and Nadeau KC (2012) The STAT5b pathway defect and autoimmunity. Front. Immun. **3**:234. doi: 10.3389/fimmu.2012.00234*
This article was submitted to Frontiers in Primary Immunodeficiencies, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 02 August 2012
doi: 10.3389/fimmu.2012.00232

APECED: is this a model for failure of T cell and B cell tolerance?

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In APECED, the key abnormality is in the T cell defect that may lead to tissue destruction chiefly in endocrine organs. Besides, APECED is characterized by high-titer antibodies against a wide variety of cytokines that could partly be responsible for the clinical symptoms during APECED, mainly chronic mucocutaneous candidiasis, and linked to antibodies against Th17 cells effector molecules, IL-17 and IL-22. On the other hand, the same antibodies, together with antibodies against type I interferons may prevent the patients from other immunological diseases, such as psoriasis and systemic lupus erythematous. The same effector Th17 cells, present in the lymphocytic infiltrate of target organs of APECED, could be responsible for the tissue destruction. Here again, the antibodies against the corresponding effector molecules, anti-IL-17 and anti-IL-22 could be protective. The occurrence of several effector mechanisms ($CD4^+$ Th17 cell and $CD8^+$ CTL and the effector cytokines IL-17 and IL-22), and simultaneous existence of regulatory mechanisms ($CD4^+$ Treg and antibodies neutralizing the effect of the effector cytokines) may explain the polymorphism of APECED. Almost all the patients develop the characteristic manifestations of the complex, but temporal course and severity of the symptoms vary considerably, even among siblings. The autoantibody profile does not correlate with the clinical picture. One could speculate that a secondary homeostatic balance between the harmful effector mechanisms, and the favorable regulatory mechanisms, finally define both the extent and severity of the clinical condition in the *AIRE* defective individuals. The proposed hypothesis that in APECED, in addition to strong tissue destructive mechanisms, a controlling regulatory mechanism does exist, allow us to conclude that APECED could be treated, and even cured, with immunological manipulation.

Keywords: AIRE, APECED, endocrine disorders, interleukin 17, interleukin 22, IPEX, T regulatory cells

INTRODUCTION

Autoimmune polyendocrinopathy syndrome type 1 (APS-1) or autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy syndrome (APECED; OMIM 240300) is a rare recessively inherited disorder (Perheentupa, 2002; Betterle and Zanchetta, 2003; Perheentupa, 2006; Husebye et al., 2009). It is caused by mutations in the autoimmune regulator (*AIRE*) gene located on locus 21q22.3 (Bjorses et al., 1996; Nagamine et al., 1997; The Finnish–German APECED Consortium, 1997). APECED displays a worldwide distribution, but specific clusters of high prevalence of the disease are observed among Finns (1:25,000; Ahonen et al., 1990) and Sardinians 1 (1:14,500; Rosatelli et al., 1998; Meloni et al., 2012). It is characterized by the variable association of autoimmune endocrine [hypoparathyroidism (HP), Addison's disease (AD), hypothyroidism, gonadal insufficiency, insulin-dependent diabetes mellitus, atrophic gastritis, and Biermer's disease] and non-endocrine disorders (keratitis, malabsorption, vitiligo, and alopecia areata) and a specific predisposition to chronic mucocutaneous candidiasis (CMC). A definite diagnosis of APECED is made upon one of the following criteria: (i) the presence of at least two of three major clinical features: CMC, HP, and AD, or (ii) one disease component if a sibling has already a definite diagnosis, or (iii) disease-causing mutations in both alleles of the *AIRE* gene. However, APECED being highly variable in its presentation, the classical triad may be complete only after years of evolution and diagnose may be therefore missed. Besides, APECED may appear during adolescence or in the young adult (Husebye et al., 2009). Therefore, criteria for a probable APECED have been defined as follows: (i) presence of one of CMC, HP, AD (before 30 years of age) and at least one of the minor components chronic diarrhea, keratitis, periodic rash with fever, severe constipation, autoimmune hepatitis, vitiligo, alopecia, enamel

Abbreviations: AADC, aromatic L-amino acid decarboxylase; AD, Addison's disease; AE, autoimmune enteropathy; APS-1, autoimmune polyendocrinopathy syndrome type 1; APECED, autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy; CMC, chronic mucocutaneous candidiasis; EECs, enteroendocrine cells; GI, gastro-intestinal; HP, hypoparathyroidism; IPEX, immune dysregulation, polyendocrinopathy, enteropathy and X-linked; IF, intrinsic factor; IL-1, interleukin-1; IL-17, interleukin-17; IL-22, interleukin-22; mTECs, thymic medullary epithelial cells; NALP-5, NACHT leucine-rich-repeat protein 5; PE, promiscuous expression; PTH, parathormone; SLE, systemic lupus erythematosus; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; TRIMs, tripartite motif-containing proteins; TSA, tissue-specific antigens.

hypoplasia, (ii) any component and anti-interferon antibodies, or (iii) any component and antibodies against NACHT leucine-rich repeat protein 5 (NALP5), AADC, tryptophan hydroxylase (TPH), or TH (Husebye et al., 2009).

FROM CIRCULATING AUTOIMMUNE ANTIBODIES TO *AIRE*, *FOXP3*, APECED, AND IPEX

Our knowledge of the nature of the condition now called APS-1 or APECED has increased simultaneously with the general development of immunology and autoimmunity. Since the condition was clearly defined in the end of 1950s and early 1960s, the characteristic clinical picture, the immunological abnormalities and the relationship to other autoimmune endocrine diseases were defined in late 1960s and early 1970s. Furthermore, the genetics of APECED, and the fact that the syndrome was caused by a recessive gene defect – as opposed to the HLA-linked genetics seen in the other solitarily occurring endocrine diseases – were characterized in the 1980s and the target antigens in the organs affected by APECED were molecularly defined in 1990s. A landmark stage in the study of APECED was reached in 1997, when the long sought APECED gene was cloned by two independent groups (Nagamine et al., 1997; The Finnish–German APECED Consortium, 1997). Finally, a new phase in APECED research occurred during the first decennium of 2000, when the autoantibodies toward soluble mediators if immune response were characterized (Meager et al., 2006; Kisand et al., 2011).

The notion that several diseases affecting endocrine organs and earlier defined as idiopathic, were in fact caused by an autoimmune response toward self antigens, became apparent when novel immunological methods became available in 1950s and early 1960s (Blizzard et al., 1963). The association of the three conditions, candidiasis, HP, and AD that were later judged to be the hallmarks of APECED was clearly stated by the groups of Blizzard and Maclaren (Blizzard et al., 1963; Brun, 1978; Neufeld et al., 1981). These groups also defined two clearly distinct syndromes with several associated autoimmune diseases: autoimmune polyglandular syndrome type 1 (PGS-1) and polyglandular syndrome type 2 (PGS-2). The nomenclature was later changed to APS-1 and APS-2, and the former further to APECED (Ahonen et al., 1990; Perheentupa, 2002, 2006; Betterle and Zanchetta, 2003).

Pioneering studies in this field were made especially with the use of immunohistochemistry, demonstrating antibodies reacting with gastric parietal cells in chronic gastritis (Walder et al., 1963; Irvine et al., 1965) and intrinsic factor (IF) in pernicious anemia (Schwartz, 1961; Jeffries et al., 1962), with thyroid epithelial cells in various forms of thyroid diseases (Witebsky et al., 1957; Irvine et al., 1962; Doniach and Roitt, 1964), with the beta cells of Langerhans islands in diabetes mellitus (Kaldany, 1979; Bottazzo et al., 1980), and adrenal cortical cells in AD (Blizzard et al., 1962).

Blizzard's group noticed that the two polyglandular syndromes, APS-1 and APS-2, differed in their HLA haplotypes (Neufeld et al., 1981). Further studies on the HLA haplotypes revealed that the genetic basis of APS-2, but also of the other isolated forms of endocrine autoimmune diseases found in APS-1, were in the HLA haplotype of the patients. In contrast, APS-1 was shown not to be linked to HLA, and studies with large patient material, collected by Perheentupa's group in Finland, clearly stated that APS-1 was linked to a recessively inherited gene defect (Ahonen et al., 1990; Perheentupa, 2002, 2006). The autoimmune endocrinopathies could thus be grouped on the basis of their genetic background in two distinct categories: those linked to HLA variation and the one, APS-1 caused by a single mutated gene (**Table 1**). At that stage, however, the responsible gene, the APECED gene, was not yet identified. Once identified, the APECED gene was renamed as *AIRE* in 1997 (Nagamine et al., 1997; The Finnish–German APECED Consortium, 1997).

Another immunopathy, termed originally as autoimmune enteropathy (AIE) and later identified as immune dysregulation, polyendocrinopathy, enteropathy and X-linked (IPEX), was described in the 1980s and 1990s (Powell et al., 1982). This disorder was later shown to be caused by a defect in a single gene, *FOXP3* (Bennett et al., 2000). IPEX and APECED are two examples of immune deficiency diseases disclosing both disturbed tolerance and autoimmune phenomena (Moraes-Vasconcelos et al., 2008). Traditionally, reviews tend to associate both IPEX and APECED because of common features. However, both clinical manifestations and predisposition to infections are rather different when comparing both diseases (Moraes-Vasconcelos et al., 2008).

AIRE GENE, MUTATIONS, AND MECHANISM OF ACTION

AIRE is expressed in thymus, lymph nodes, and fetal liver, and encodes a protein with two putative zinc fingers and other motifs suggestive of a transcriptional regulator (Nagamine et al., 1997; The Finnish–German APECED Consortium, 1997). The *AIRE* gene, approximately 13 kb in length, contains 14 exons that encode a polypeptide of 545 amino acids. The AIRE protein functions as a transcription factor (Fierabracci, 2011; Gardner et al., 2009). AIRE is expressed in the thymic medullary epithelial cells (mTECs, **Figure 1**) and in cells of the monocyte/dendritic cell lineage (Kogawa et al., 2002). mTECs through the expression of MHC class II express a wide array of tissue-restricted antigens (TRAs) derived from different organs in the body. TRAs include self-proteins with patterns of expression restricted to a single or small handful of organs. Thymic expression of TRA serves as an important source of self-antigens to allow the negative selection of autoreactive T cells. Collectively, mTEC and thymic monocyte/dendritic cells play a crucial role in establishing self-tolerance by eliminating autoreactive T cells (negative selection) and/or by producing immunoregulatory FOXP3$^+$ T cells, which prevent CD4$^+$ T cell-mediated organ-specific autoimmune diseases. Collectively, several studies in mouse and man have shown that AIRE regulates thymic expression of several genes of ectopic peripheral proteins including many TRAs. Thus, AIRE dysfunction leads to a decrease in the expression of TRAs in the thymus, and consequently, autoreactive T cell clones escape into the periphery (Derbinski et al., 2005; Moraes-Vasconcelos et al., 2008; Gardner et al., 2009; Fierabracci, 2011)

The most common *AIRE* mutation, the "Finnish mutation," R257X, affects 82% of Finnish *APECED* alleles (Nagamine et al., 1997; The Finnish–German APECED Consortium, 1997). Interestingly, this mutation occurs also in 70% of the Russian APECED patients studied (Orlova et al., 2010). The same mutation, R257X was also detected in Swiss patients on a different haplotype with closely linked polymorphic markers (Nagamine et al., 1997) and in

Table 1 | Key laboratory findings in the different autoimmune endocrine diseases.

Diagnosis	Clinical findings	Autoantibodies	HLA	Gene defect	Cellular immune response
APECED (APS-1)	Candidiasis and multiple failure of most endocrine organs and non-endocrine autoimmunity	Against all affected organs	No association (?)	Close to 100 mutations described in the *AIRE* gene	CTL against affected organs? Failure in Treg population
APS-2	Addison's disease with insulin-dependant type I diabetes or thyroid diseases	Against adrenal cortex, pancreatic beta cells, thyroid	Risk haplotypes HLA DR3: DRB1*0301, DQA1*0501, DQB1*0201 DR4 DR1, DR7, DR13, and DR14: protective (Betterle and Zanchetta, 2003)	No single gene defect	CTL against affected organs?
Addison's disease	Low levels of gluco- and mineralocorticoid High ACTH, low cortisol, high renin, low aldosterone, subnormal cortisol response to ACTH test: hyponatremia, hyperkalemia	Against P450c21, P450scc	HLA-DRB1-DQA1-DQB1 HLA-DR3	No single gene defect	CTL against affected organs?
IPEX	Enteropathy, diabetes skin disease (mainly eczema), failure to thrive, thyroiditis, recurrent infections	Against enterocytes (autoimmune enteropathy-related 75-kDa antigen) pancreatic-islet cells, insulin, and glutamic acid decarboxilase (GAD), and thyroid (antithyroid microsomal antibodies)	No association	Defective *FOXP3* gene	Impaired function of regulatory T cells, defective IL-2, IFN-γ, and TNF-α production. Increased production of IL-17

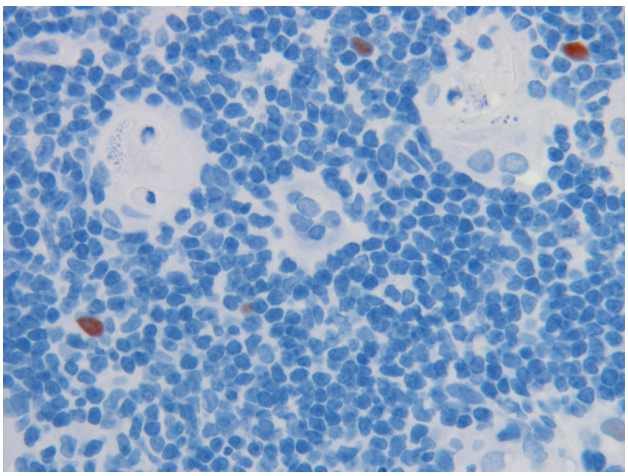

FIGURE 1 | Medullary epithelia cells in thymus, expressing the AIRE proteins (reddish brown), in close vicinity of the Hassall's corpuscles (HC) where auto-reactive T cells are thought to be destroyed. Note cell debris in HC. Magnification 1:40. AIRE was demonstrated with specific monoclonal antibody at 1:2,000 dilution.

northern Italian *APECED* patients. Nonsense mutation R139X was found as the predominant haplotype among Sardinian patients (18/20 independent alleles; Rosatelli et al., 1998). Other hotspots have been identified such as the Y85C missense mutation in an isolated Iranian Jewish community (Zlotogora and Shapiro, 1992; Björses et al., 2000). A 13-bp deletion in exon 8 [1085–1097(del)] is ubiquitous and can be found in Norwegians, but also Anglo–Saxons descendant (Zlotogora and Shapiro, 1992) and south Americans (Moraes-Vasconcelos et al., 2008). Today, over 60 different mutations have been described throughout the coding region of AIRE (Akirav et al., 2011).

CLINICAL PICTURE OF APECED

The clinical picture of APECED is characterized by sequentially occurring diseases, with great variation among the patients as to the severity and time course of the various conditions. In most cases, the affected individual starts suffering from CMC in early infancy or childhood. In most cases, the next organs to be affected are the parathyroid glands, followed by AD and at puberty, hypogonadism mainly in female teens or young adults. Additional clinical features are less common, and include diabetes type I, hypothyroidism, atrophic gastritis with or without pernicious anemia (Biermer's disease), cutaneous manifestations (alopecia areata, vitiligo, transient skin rash during fever episodes, non-infectious nail dysplasia), ocular symptoms (keratoconjunctivitis, dry eye, iridocyclitis, cataract, retinal detachment, and optic atrophy; Merenmies and Tarkkanen, 2000), enamel dysplasia, hyposplenism/asplenia (implying vaccination against *Streptococcus pneumonia, Haemophilus influenzae*, and Hepatitis B as well as antibiotic prophylaxis), autoimmune hepatitis, tubulo-interstitial nephritis, or organized pneumonitis. Involvement of the gastrointestinal (GI) tract may be responsible for chronic diarrhea, constipation, and malabsorption leading sometimes to malnutrition. GI involvement is difficult to assess as it can be due to numerous various causes that may be associated or follow each other during the life of the patients.

CANDIDIASIS

Chronic mucocutaneous candidiasis infection by *Candida albicans* is one of the major characteristic of APECED, usually one of first symptoms and most likely the most disabling features of APECED. CMC is naturally not specific for APECED but any child with CMC should be suspected of APECED. According to the Finnish experience, almost all adults with APECED display symptoms of CMC, up to 70% of the patients at the age of 10, up to 94% at age of 20, and 97% at the age of 30 (Perheentupa, 2002, 2006; Betterle and Zanchetta, 2003; Husebye et al., 2009). However, the course and severity vary widely. Oral candidiasis affects the tongue, the buccal mucosa, the gingival, and the pharynx. It ranges in severity from mild form with redness, soreness, angular cheilitis, pseudomembranous lesions, erosions, ulceration and pain to severe chronic inflammation with dysphagia, and development of hyperkeratotic plaques. In the absence of active antimycotic treatment and careful follow-up, chronic oral candidiasis may lead to the development of squamous cell carcinoma with potential lethality by metastatic dissemination. *Candida* esophagitis has been reported to affect 15–22% of the patients (Perheentupa, 2006; Kisand et al., 2011) with pain while swallowing, retrosternal pain, and dysphagia (Ahonen et al., 1990; Husebye et al., 2009). Chronic esophagitis can lead to local stricture and exceptional esophageal cancer (Rautemaa et al., 2007).

Intestinal candidiasis may cause chronic diarrhea. It should be stressed that esophageal and intestinal candidiasis may occur without any active oral candidiasis. Genital candidiasis affects mainly women with pruritus and vaginal whitish discharge while genital candidiasis seems less frequent in males, possibly underreported due to discrete signs of balanitis. Lastly, *Candida* may affect the nails with chronic paronychia and onychomycosis (Collins et al., 2006). Fingernails are more commonly affected than toenails and the thumbs are the commonest digit affected. This can be explained as infection occurs during the "thumbsucking" period. Management of candidiasis in APECED patients implies an excellent oral hygiene with a careful and regular dental follow-up. Candidiasis should be treated aggressively with antimicrobial therapy and regular prophylaxis should be given.

Any clinically suspicious, chronic thickening or erosion of the mucosa that does not heal should be biopsied to rule out a potential underlying lesion of squamous cell carcinoma. Any difficulties in swallowing or eating or retrosternal pain should prompt to perform esophagoscopy (Rautemaa et al., 2007).

HYPOPARATHYOIDISM

Hypoparathyroidism is one of the first endocrine features of APECED. Symptoms are related to hypocalcemia, muscle cramps, paresthesia, clumsiness, seizures, and diarrhea. The diagnosis is simply based on blood calcium, phosphorus, and parathormone (PTH) levels: hypocalcemia, hyperphosphatemia, inadapted normal/low PTH without any kidney failure. It is considered that APECED should be systematically considered in cases of primary HP (Husebye et al., 2009). Antibodies against NALP5 as well as

against the calcium-sensing receptor of parathyroid epithelial cell have been identified in APECED patients (Gavalas et al., 2007; Kemp et al., 2009, 2010). Patients who are free of HP need an annual monitoring of blood calcium and phosphorus levels. Management of HP relies on daily oral supplementation of vitamin D derivatives and calcium.

GASTRITIS AND PERNICIOUS ANEMIA

Chronic gastritis, with or without concomitant pernicious anemia belongs to the APECED complex but is found only in a fraction of cases. In non-APECED population, two types of chronic gastritis occur, divided by Strickland into type A and B gastritis. Type A gastritis was known to be caused by autoimmunity while the B gastritis was suspected to be the results of environmental factors. In early 1980s, it was shown by Warren and Marshall (1984) that the major environmental factor was in fact a chronic infection with *Helicobacterium pylori*.

The type A chronic gastritis, with and without pernicious anemia that occur in non-APECED individuals, is clearly linked to certain HLA risk haplotypes, in analogy to isolated AD. In APECED patients, the chronic gastritis differs from the above also in time of occurrence and the speed of the progression. In non-APECED patients, the time needed for progression from the early stage of gastritis, the superficial form to diffuse gastritis, to atrophic gastric and to full gastric atrophy is a slow process, taking up several decennia. Also, the process usually starts in the adult life. In contrast, an APECED-associated gastritic process is much faster and can start in the first decennium of life. Thus, one of the authors of this review was able to follow such a gastric process in two 8-year-old girls with sequential gastric biopsies and could see how, within the time period of only 2 months, the superficial process lead to complete gastric atrophy of the fundus and corpus (K. Krohn, personal experience).

The target molecule for the parietal cell antibodies were shown to be the sodium-potassium channel molecule of the parietal cells on corpus and fundal part of the stomach (Karlsson et al., 1988). In antral gastritis, the antigen are the gastrin-producing cells (Uibo and Krohn, 1984).

Pernicious anemia is the end stage of the gastric immunological destruction, caused partly by the lack of IF, that in addition to the hydrochloric acid is the main product of parietal cells, but also by the autoantibodies recognizing this vitamin B12-binding protein. There are two types of antibodies to IF: one blocking vitamin B12 binding to IF and another type, binding to the IF molecule without interfering with vitamin B12 binding (Toh et al., 1997). Both antibody types prevent the binding of IF to its receptor on the ileal mucosa and subsequent translocation of the vitamin B12 from ileum to circulation.

ADDISON'S DISEASE

Adrenocortical failure or AD, described by Thomas Addison in the ninetieth century, is considered one of the three hallmarks of APECED, but it occurs also as a solitary disease, or as part of the APS-2 complex. Today, in western word, most cases of AD are caused by autoimmunity, but adrenal cortical destruction and subsequent cortical failure can be caused by several other factors, notably by secondary tuberculosis or other chronic infections. In retrospect, the cases described by Thomas Addison were most likely caused by tuberculosis.

The clinical signs and symptoms of AD are mostly similar in APECED and in solitary AD as well as in APS-2 complex. These include decreased levels of gluco- and mineralocorticoids and elevated ACTH concentrations. The most severe consequence of AD is the life-threatening Addisonian crisis, characterized by general fatigue, dizziness, diarrhea, and death, if the patient is not quickly substituted with corticosteroids, mainly hydrocortisol.

Autoantibodies to adrenal cortex are the characteristic immunological feature in AD, be it part of APECED or APS-2 or the solitary form. These antibodies can be easily demonstrated by immunofluorescence. However, in APECED, but not in the other forms of AD, the autoantibodies are precipitating, and this phenomenon can be demonstrated by Ouchterlony's immunodiffusion (Andrada et al., 1968; Krohn et al., 1974; Heinonen et al., 1976). In immunodiffusion with APECED serum against adrenal homogenate three precipitating lines were observed, and one of these were shown to represent a mitochondrial antigen while the two others were microsomal.

The nature of the adrenal cortical autoantigens were revealed in early 1990s and shown to be the three main steroidogenic enzymes, P450c17, P450c21, and P450scc (Krohn et al., 1974; Winqvist et al., 1993; Uibo et al., 1994a,b). These three enzymes were also shown to be the ones that could be precipitated by immunodiffusion. The autoantibodies against these three steroidogenic enzymes clearly distinguish the three clinical conditions with adrenal failure: antibodies to all three can be found only in APECED, while in solitary AD and in APS-1, only antibodies to P450c21 are seen. Furthermore, in non-autoimmune AD, caused by tumors or chronic infections, such antibodies do not occur.

GONADAL FUNCTIONS

Autoimmune oophoritis is responsible for an ovarian insufficiency that may be dramatic for female patients as insufficiency starting in teenagers and young adults. Patients may have either a primary amenorrhea with no or arrested puberty. Other patients develop premature menopause. The diagnosis is confirmed by sexual hormones status; elevated plasma levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) and low estrogen levels. Autoantibodies against side-chain cleavage enzyme have been related to ovarian insufficiency (Soderbergh et al., 2004) and also steroidogenic enzymes antibodies against cytochrome p450 21-hydroxylase (CYP21A2), cytochrome p450 17α-hydroxylase (CYP17), and cytochrome p450 side-chain cleavage enzyme (CYP11A1).

In female patients, hormonal substitution by estrogen needs to be initiated during puberty. It is strongly advised not to delay pregnancy. In case of hypogonadism, embryo donation has been tried with success.

In males, testicular failure is less common and occurs later. The prevalence of hypogonadism in males is three times lower (8–28%) than in females (35–70%; Perheentupa, 2006). It leads to clinical hypogonadism or isolated azoospermia (Husebye et al., 2009). It has been hypothesized that the blood–testis barrier protects the Leydig cells from an autoimmune attack. However,

the physiopathogenic link between circulating autoantibodies and hypogonadism is far from being clear. The two steroidogenic enzymes, p450scc and p450c17, are the main antigens in gonadal failure linked to APECED, but other potential antigenic targets have been identified such as testis-expressed protein TSGA10 (Reimand et al., 2008). However, despite autoantibodies directed against TSGA10 in 7% of the APECED patients, no correlation could be found with gonadal failure (Reimand et al., 2008). One should not forget that the origin of gonadal dysfunction may be related to an authentic-specific autoimmune attack but also be related to other hormonal dysfunction such as AD, pituitary insufficiency, dysthyroidism, or diabetes for instance. Besides, Schaller et al. (2008) suggested that lack of AIRE might affect fertility by disrupting scheduled apoptosis of testicular germ cells. In this respect, the recent hypothesis presented by Matsumoto (2011) that the function of AIRE in thymus would not be in the regulation of transcription but rather in the development and differentiation of the medullary epithelial cells is of primary interest

OTHER ENDOCRINE DISORDERS

Various other endocrine disorders have been described such diabetes type I mellitus, hypothyroidism, and pituitary failure, the latter diagnosed by a growth hormone deficiency. The diagnosis and management of these conditions does not differ from the standard guidelines for each disorder separately (Perheentupa, 2002; Betterle and Zanchetta, 2003; Husebye et al., 2009).

OTHER NON-ENDOCRINE DISORDERS

Enamel hypoplasia affect mainly permanent teeth (Perheentupa, 2006), but also deciduous teeth (Pavlic and Waltimo-Sirén, 2009). Pavlic and Waltimo-Sirén (2009) recently suggested that an inadequate process of enamel formation might affect all ameloblasts in phase. Ameloblasts have an epithelial origin with parenchymal cells of endocrine origin. It is speculated that ameloblasts or secreted protein in the extracellular matrix may be the target of autoantibodies leading to hypoplasia. Thereby, APECED would be the first model of dental hard tissue autoimmune disease (Pavlic and Waltimo-Sirén, 2009).

Ocular manifestations affect 25% of the patients and include mainly keratitis that can lead to blindness. It is assumed that the origin of keratitis is the result of autoimmunity against corneal epithelium (Merenmies and Tarkkanen, 2000; Perheentupa, 2006). However, to our knowledge no specific antibodies have been identified in APECED patients. Only antibodies against OBP1 have been found in the AIRE mouse model against lacrimal glands (DeVoss et al., 2010).

Hyposplenism or asplenia is often diagnosed upon the development of thrombocytosis, circulating Howell–Jolly bodies, abdominal ultrasound imaging or in case of severe *S. pneumoniae* infection (Pollak et al., 2009). Destruction of the spleen in APECED has been related to an autoimmune attack against the spleen (Perheentupa, 2002, 2006; Betterle and Zanchetta, 2003; Husebye et al., 2009) although the exact mechanism remains obscure. Again, the mechanisms proposed by Schaller et al. (2008) and by Matsumoto (2011) are of interest, as AIRE expression has also been described in lymphoid tissue and skin. A speculative hypothesis to the evolution of splenic atrophy could thus be disturbance of differentiation, due to lack of AIRE expression.

Various types of GI manifestations are common in APECED patients. These include chronic diarrhea that can be related to HP, severe constipation. Intestinal infection by candida and giardia especially, pancreatic insufficiency and autoimmune enteropathy. Several autoreactive circulating antibodies directed toward intestinal components have been described. Ekwall et al. (1998) identified TPH as an intestinal autoantigen in APECED patients. TPH is expressed in serotonin-producing cells in the central nervous system and in the intestine. In their series of 80 patients, they were able to relate "GI symptoms" to the presence of circulating TPH antibodies and also to the total absence of enterochromaffin cells in the mucosa of small bowel. These enteroendocrine cells (EECs) are scattered through the intestinal mucosa, from the gastric body and antrum to the rectum. They play a key role in growth of the gut, blood flow, motility, secretion of pancreatic enzymes, bile, and bicarbonate-rich fluid (Posovszky et al., 2012). TPH antibodies were found in 89% of the APECED patients with GI symptoms and in 34% of those without (Ekwall et al., 1998). Antibodies can precede clinical symptoms (Ekwall et al., 1998). Conversely, TPH autoantibodies are absent in other inflammatory or autoimmune intestinal diseases. Additionally, Sköldberg et al. (2003) identified also autoantibodies against histidine decarboxylase expressed by EEC – like cells in the gastric mucosa. It is noteworthy, that it is not a routine procedure to perform EECs staining on intestinal biopsies in case of diarrhea or malabsorption, as stressed by Ohsie et al. (2009). Besides, several studies showed repeatedly that EECs were lacking in the intestinal mucosa and were related to chronic diarrhea (Padeh et al., 1997; Ward et al., 1999; Oliva-Hemker et al., 2006; Posovszky et al., 2012).

Tubulo-interstitial nephritis, life-threatening autoimmune bronchiolitis and other rare manifestations have also been reported in APECED (Perheentupa, 2002, 2006; Betterle and Zanchetta, 2003; Husebye et al., 2009). The main identified autoantibodies are summarized in **Table 2**.

TREATMENT

Management of APECED relies in education of the patients to know his disease, education of the local physician, and the knowledge that new components of the disease may develop during life. Psychological support is strongly recommended as this disease impairs greatly the quality of life of the patients (Perheentupa, 2006). Except candidiasis treatment that has been explained previously, treatment of APECED relies mostly on hormone replacement therapy according to affected organs (thyroid, parathyroid, pancreas, etc.). In some rare and potentially lethal situations, however, patients may require corticosteroid treatment in association with immunosuppressive therapies. These rare situations include autoimmune hepatitis, especially its fulminant form, which may be lethal and therefore prompt immunosuppressive therapy is needed (Obermayer-Straub et al., 2001). The same is true for interstitial nephritis and bronchiolitis in association to APECED. Immunosuppressive therapies have been also proposed in case of severe intestinal malabsorption with efficacy (Padeh et al., 1997; Ward et al., 1999). Very recently, Rituximab, a chimeric monoclonal antibody targeting B cell lymphocytes

Table 2 | Main identified target of autoimmune antibodies in APECED patients.

Diagnosis	Main identified circulating autoantibodies
Addison's disease	21 hydroxylase, 17α hydroxylase
	Side-chain cleavage enzyme antibodies
	(or steroid cell antibodies)
Hypoparathyroidism	NALP5, Ca^{2+} sensing receptor
Hypothyroidism	Thyroperoxydase
	Thyroglobuline
Hypogonadism	17α hydroxylase
	Side-chain cleavage enzyme antibodies
	(or steroid cell antibodies)
Diabetes type I	Glutamic acid decarboxylase 65-kDa isoform
	(GAD65)
	Insulin
	Tyrosine phosphatase (IA2)
Pituitary insufficiency	Tudor Domain containing protein 6 (TDRD6)
Atrophic gastritis/	Intrinsic factor, gastric parietal cell
Biermer's disease	
Intestine	Glutamic acid decarboxylase 65-kDa isoform
	(GAD65)
	Histidine decarboxylase
	Tryptophan hydroxylase
Autoimmune hepatitis	Aromatic L-amino acid decarboxylase (AADC)
	Cytochrome P450 1A2
	Cytochrome P450 2A6
	Cytochrome P450 1A1
	Cytochrome P450 2B6
Vitiligo	Transcription factors: SOX 9, SOX 10, aromatic
	L-amino acid decarboxylase (AADC)
Alopecia areata	Tyrosine hydroxylase
Nephropathy	Antibody against tubular basement membrane
	(Hannigan et al., 1996)
Pulmonary disease	Potassium channel regulatory protein (KCNRG)
Eye	OBP1*
Non-tissue specific**	IFN-α, IFN-β, IFN-ω, IL-22, IL-17F, IL-17A

*Identified in a mouse model $AIRE^{-/-}$.
**Main non-tissue-specific antibodies according to Kisand et al. (2011).

expressive CD20 has been successfully used in a young patient with bronchiolitis (Popler et al., 2012). The rationale for Rituximab use in APECED is supported by the presence of B cell infiltrates in the affected organs (Gavanescu et al., 2008).

AUTOANTIBODIES TOWARD INTERFERONS AND CYTOKINES

At the beginning of this millennium, the antibody responses to the main target organs affected in APECED, and the responsible target antigens were fairly well characterized. A new period in APECED studies started along the publication by Meager et al. (2006), describing high-titer antibodies to several type I interferons in practically all APECED patients studied. This anti-interferon response was exceptionally strong, since serum titers up to 1:1,000,000, and clearly exceeding the titers seen against organ-specific antigens, were found.

Furthermore, high-titer antibodies were seen against the two main mediators secreted by Th17 cells, interleukin-17 and interleukin-22 (IL-17 and IL-22). Responses with lower titers were occasionally seen against other interleukins, too. In our own as yet unpublished observations we have detected occasional high-titer responses against several other interleukins and chemokines, as well, but in contrast to the aforementioned responses, these responses are not characteristic to all APECED patients but rather occur occasionally in only a few patients.

The significance of these novel findings are still unclear, but some information concerning the role of IL-17/IL-22 antibodies in the chronic candida infections, characteristic for APECED, has been obtained. Th17 cells secrete IL-17 and IL-22, which are cytokines with potent antifungal properties (Engelhardt and Grimbacher, 2012) and the occurrence of autoantibodies against IL-17/IL-22 were reported to closely correlate to the presence of candida infection (Kisand et al., 2011; Engelhardt and Grimbacher, 2012). However, recent evidence points to a new interaction between AIRE and dectin-1, a pattern-recognition receptor that is important in antifungal innate immunity. Pedroza et al. (2012) recently showed that AIRE participates in the dectin-1 signaling pathway, and thus, missing AIRE activity could contribute to fungal susceptibility through this pathway. Dectin-1 is expressed on phagocytes and was recently shown to induce a non-canonical caspase-8 inflammasome in response to fungal and mycobacterial infection (Gringhuis et al., 2012). The activation of the dectin signaling pathway also leads to expression of IL-17 and 22 and defensins, however. Besides, other mechanisms such Dominant-negative mutations in STAT3, gain of mutation of STAT1, mutations in IL-17F and IL-17R may be alternate causes of CMC (Engelhardt and Grimbacher, 2012).

The significance of the antibody response toward interferons and other cytokines is presently also unclear. One could speculate that some of these antibodies against type I interferons as well as reacting with IL-17 and IL-22 might have a protective function. As pointed out by Waterfield and Anderson (2011), antibodies to type I interferons do not seem to lead to increased susceptibility to viral infections. This resistance might be due to redundancy and it has to be seen whether this anti-interferon response is directed only toward certain members of the interferon family. While Th17 cell response and the release of soluble IL-17 and IL-22 are evidently necessary for the defense against mucocutaneous candida infection, the same cytokines have a role in the development of psoriasis. Similarly, interferons are known to be involved in the pathogenesis of several conditions, and one such chronic ailment is the autoimmune diseases belonging to the systemic lupus erythematosus (SLE) complex. Anti-interferon alpha antibodies are currently being tested as a therapeutic mean against SLE (Merrill et al., 2011). In order to be able to find out if the antibodies against interferons and other cytokines could have a protective role in APECED, large APECED patient cohorts have to be studied in order to find out whether, e.g., psoriasis and SLE are

significantly less common in APECED patients than in the general population.

The reason for the antibody response toward soluble immune mediators is still unclear, and we do not yet know what exactly elicits them and thus, only speculative scenarios can be presented. It is conceivable to hypothesize, however, that the tissue destruction preceding the failure of the endocrine organs may have a role. Tissue destruction, be it caused by trauma, viral infection or autoimmune attack, would probably lead not only to the release of potential tissue-specific autoantigens and thus, to autoantibody formation against these proteins, but could also lead to an inflammatory response and production of several mediators of inflammation. One key group of molecules in this respect is the acute phase proteins, notably those belonging to the IL-1 group.

It is generally believed that the destruction of the endocrine organs in APECED is caused by the autoreactive $CD8^+$ cytotoxic T cells, although definitive evidence for this mechanism is still lacking (Betterle and Zanchetta, 2003; Moraes-Vasconcelos et al., 2008). This hypothesis is reinforced by the examination of microscopic examinations of samples, sometimes obtained post-mortem. Indeed, parathyroid, adrenal glands, or ovaries pathology disclosed also atrophy and lymphocytic infiltration that suggest lymphocytic aggression of the organs leading to atrophy and dysfunction (Betterle and Zanchetta, 2003). This is also stressed, indirectly, by the analysis of the AIRE-deficient mouse model, who develop also a lymphocytic infiltration in some inner organs along with atrophy (Ramsey et al., 2002).

However, cell destruction caused by an immune response against the endocrine organ would in fact lead to a similar situation that is thought to happen in viral infections. In fact, several autoimmune diseases, such as diabetes type I or chronic autoimmune liver diseases are thought to be a consequence of preceding viral infection: enterovirus infection in the case of diabetes type I and hepatitis B in the case of chronic active hepatitis. In viral infections, a specific group of intracellular regulatory molecules, TRIMs (tripartite motif-containing proteins), have been shown to have a key role in eliciting an autoimmune or auto inflammatory consequence (Jefferies et al., 2011).

The TRIM protein family is a form of RING domain containing E3 ligases and they exert a variety of biological functions, related to immunity and inflammation (Jefferies et al., 2011). Specifically, of the more than 20 different TRIM proteins, some seem to up-regulate the expression of type I interferons and proinflammatory cytokines, notable interleukin-1beta (IL-1beta). Furthermore, the same mediators of immune response and inflammation are in some cases known to up-regulate the expression of TRIMs. Thus, a vicious circle can theoretically occur and this in turn could lead to autoimmunity. So far, overexpression of TRIMs, or an autoimmune response toward them, has been shown to be linked to autoimmune and autoinflammatory processes in Sjögren's syndrome or rheumatoid arthritis (Jefferies et al., 2011).

Presently, we have no information how the occurrence of autoantibodies toward the interferons and other mediators of immune response might affect the aforementioned vicious circle, but it is conceivable to speculate that such an antibody response could have an balancing effect. One could thus form a hypothesis, that in APECED, the primary defect outside thymus, where the autoreactive T cells are not destroyed, would be the cell destruction of the endocrine organs by cell-mediated immune response, followed by release of cellular components taken up by professional antigen presenting cells and further stimulating the activation of $CD4^+$ Th-cells and finally resulting in an autoantibody response to these organ-specific antigens. However, simultaneous overexpression of TRIMs and subsequent up-regulation of a variety of soluble mediators of immune response and inflammation, such as interferons and members of the IL-1 family would lead to autoantibody formation also against these cytokines. Lastly, one reason for the break of tolerance to immune mediators, and subsequent production of autoantibodies could be related the fact that AIRE expression seems to occur, in addition to thymic epithelial cells also outside thymus, notably in dendritic cells, that normally express also such mediators (Heath and Carbone, 2009)

The consequences of such cytokine-directed antibody response are still an open question. In case of the Th17 type interleukins (IL-17 and IL-22) there is convincing evidence that such antibodies are linked to the CMC. However, at least in some cases, the antibodies may have a balancing, down-regulating effect on the expression of the corresponding biologically active molecules but also, by regulating the immune response to target organs. Thus, it is possible to presume, that especially the antibodies to type I interferons might have a protective effect, as some chronic immune diseases, such as psoriasis and SLE, are rare or non-existing among APECED patients.

CELL-MEDIATED IMMUNE RESPONSES

Although it is now a generally accepted view that the consequence of the AIRE defect in APECED will lead to the escape of the potentially autoreactive T cells, there is in fact rather little direct evidence to show that the tissue destruction in the endocrine organs affected in APECED is caused by cytotoxic $CD8^+$ T cells. Furthermore, most studies describing the phenotype of the lymphocytes infiltrating affected organs is not from APECED patients directly, but from patients suffering of solitary lesions that are similar to the ones seen in APECED, such as solitary AD or diabetes. However, the solitary endocrine diseases, such as isolated thyroid disease or AD are remarkably similar in their clinical picture as well as immunological findings as those of APECED. Thus, in solitary AD and in APECED with adrenocortical failure, autoantibodies recognize the p450c21 steroidogenic enzyme. Interestingly, in this disease complex $CD8^+$ T cells that reach against specific T cell epitopes in p450c21 has been demonstrated (Bratland et al., 2009; Rottembourg et al., 2010) Likewise, in thyroid diseases, thyroglobulin and thyroid peroxidase are recognized by the autoantibodies, irrespective if the condition is occurring alone, in association of APS-2 or as part of the APECED complex. The similar synergism in terms of the nature of autoantigens occurs in chronic immunological liver diseases, too.

In chronic aggressive hepatitis the lymphocytic infiltrating cell population has been shown to be of the $CD8^+$ lineage (Si et al., 1984). In a murine model of Graves' disease, the $CD8^+$ cell population contains also the recently identified $CD8^+CD122^+$ T cells that are functionally similar to the $CD4^+CD25^+$ regulatory T cells (Ryan et al., 2005). Furthermore, studies in thyroid and

other affected organs show that one of the main cell population in the lymphocytic infiltrate are in fact the $CD4^+$ Th17 cells that secrete as effector molecules, the cytokines IL-17 and IL-22. In experimental autoimmune diseases, the balance between the Th17 effector cells and the two regulatory T cells, $CD8^+CD122^+$ and $CD4^+CD25^+$, seems to regulate both the occurrence and severity of tissue destruction and functional failure.

There could thus be two distinct mechanisms operating in the pathogenesis of autoimmunity in the endocrinopathies: one mediated by soluble effector molecules, such as IL-17 and IL-22 as well as type I interferons, and an other one mediated by effector T cells, which are either of the $CD8^+$ CTL cell or of the Th17 effector cell lineage. To counteract these, again two distinct biological processes would occur: the production of autoantibodies and secondly, the emergency of the regulatory T cells. As to the regulatory T cell response, it is to note that one key immunological failure in APECED, is the dysregulation of the Treg cell maturation (Ryan et al., 2005; Kekäläinen et al., 2007; Saitoh et al., 2007; Laakso et al., 2010, 2011; Wolff et al., 2010).

In normal thymus, Treg maturation follows a preprogrammed scheme, and the immature $CD8^+CD4^+FOXP3^+$ seems to be prone to apoptosis, whereas the more mature form $CD4^+CD8^-FOXP3^+$ cells form the active Treg population (Lehtoviita et al., 2009). According to Endharti et al. (2011) the $CD8^+CXCR3^+$ Tregs in humans are functionally similar to murine $CD8^+CD122^+$ Tregs. Furthermore, in APECED patients the recent thymic emigrant (RTE) pool of Treg cells shift to the activated pool and the RTE reservoir is depleted. Most importantly, in APECED patients these cells express less FOXP3 than in the healthy controls (Laakso et al., 2010). Thus, in APECED the newly formed Treg cells have a developmental defect and their function is therefore impaired. Data concerning the $CD8^+$ regulatory T cells in APECED patients is missing, however.

The finding that the regulatory T cell population in APECED is functionally defective and that the expression of the key molecule for Treg function, the FOXP3 is impaired, is consistent with clinical findings in IPEX syndrome, caused by a defect in the function of the FOXP3 gene. However, it should be noted that the effect of FOXP3 mutations in Treg population also in the IPEX patients is highly variable. Also, in contrast to APECED there seems to be a genotype–phenotype correlation in IPEX, as different mutations are associated in variable clinical picture, that show differences in severity as well in the types of clinical components that are present (Torgerson et al., 2007; d'Hennezel et al., 2009) A consistent finding in IPEX is however the inability of the $CD4^+CD25$ high Tregs to suppress the function of autologous effector T cells (Bacchetta et al., 2006). There are, thus, several differences in the clinical picture of APECED and IPEX, but both conditions show clear immune destruction of at least some endocrine organs. Both conditions also share some similarities in the GI symptoms.

The proposed hypothesis that in APECED both tissue destructive mechanisms and controlling regulatory mechanisms exist raises a question whether APECED could be treated or even cured by immunological manipulations. To find an answer for this question is one of the further challenges for APECED research

ACKNOWLEDGMENTS

The study was financially supported by the European Science Foundation (ESF) and the Sigrid Juselius foundation (to Nicolas Kluger). We thank Professors Jaakko Perheentupa, Adrian Hayday, and Pärt Peterson, as well as Drs Kai Kisand, Annalisa Macagno, and Edward Stuart for stimulating discussions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 April 2012; accepted: 15 July 2012; published online: 02 August 2012.
Citation: Kluger N, Ranki A and Krohn K (2012) APECED: is this a model for failure of T cell and B cell tolerance? Front. Immun. ***3****:232. doi: 10.3389/fimmu.2012.00232*
This article was submitted to Frontiers in Primary Immunodeficiencies, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

PERSPECTIVE ARTICLE
published: 18 July 2012
doi: 10.3389/fimmu.2012.00210

Pathogenesis of autoimmunity in common variable immunodeficiency

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Common variable immunodeficiency (CVID) presents in up to 25% of patients with autoimmune (AI) manifestations. Given the frequency and early onset in some patients with CVID, AI dysregulation seems to be an integral part of the immunodeficiency. Antibody-mediated AI cytopenias, most often affecting erythrocytes and platelets make up over 50% of these patients. This seems to be distinct from mainly cell-mediated organ-specific autoimmunity. Some patients present like patients with AI lymphoproliferative syndrome. Interestingly, in the majority of patients with AI cytopenias the immunological examination reveals a dysregulated B and T cell homeostasis. These phenotypic changes are associated with altered signaling through the antigen receptor which may well be a potential risk factor for disturbed immune tolerance as has been seen in STIM1 deficiency. In addition, elevated B cell-activating factor serum levels in CVID patients may contribute to survival of autoreactive B cells. Of all genetic defects associated with CVID certain alterations in TACI, CD19, and CD81 deficiency have most often been associated with AI manifestations. In conclusion, autoimmunity in CVID offers opportunities to gain insights into general mechanisms of human autoimmunity.

Keywords: autoimmune cytopenia, autoimmunity, CD21^{low} B cells, common variable immunodeficiency, hypogammaglobulinemia

Autoimmunity is an integral part of immune dysregulation in a quarter of patients with common variable immunodeficiency (CVID), often presenting as the first manifestation of the disease (Agarwal and Cunningham-Rundles, 2009). In recent years analyses of the immune disturbances have revealed complex dysregulations of the immune system. In parallel, progress in our comprehension of the pathogenesis of connective tissue disorders like systemic lupus erythematosus (SLE) allows for comparison of common roots of human autoimmune (AI) disorders.

This perspective article is an attempt to summarize the factors which contribute to autoimmunity in CVID.

Autoimmune cytopenias are the most common AI manifestations in CVID and the focus of this article. In the context of distinct associated alterations of the cellular immune system AI cytopenias appear to be a separate manifestation from organ-specific autoimmunity in CVID (Boileau et al., 2011; Cheng and Anderson, 2012). The presentation of AI-CVID patients resembles patients with autoimmune lymphoproliferative syndrome (ALPS) with the coincidence of lymphoproliferation and AI cytopenias (Seve et al., 2008; Wehr et al., 2008; Boileau et al., 2011). While none of the cellular markers, such as increased double negative T cells or reduced switched memory B cells, helped to distinguish AI-CVID from FAS-ALPS, increased serum levels of soluble Fas ligand, interleukin (IL) 10, and vitamin B12 allowed a distinction between FAS-ALPS patients and AI-CVID to be made (Rensing-Ehl et al., 2010). None of the tested CVID patients carried a genomic or somatic mutation in FAS, rendering FAS-ALPS a differential diagnosis. Thus, the reason that lymphoproliferation and autoimmunity are seen together in most of the CVID patients remains obscure. Other causes of ALPS and ALPS-related disorders have not been excluded systematically in AI-CVID.

Other immunodeficiencies strongly associated with AI manifestations comprise immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome, autoimmune polyendocrine syndrome type 1, combined immunodeficiencies (CID) including hypomorphic severe (S)CID variants (Liston et al., 2008), both calcium channelopathies, Wiskott–Aldrich syndrome (WAS), DiGeorge syndrome, Good syndrome, activation-induced deaminase (AID) deficiency, CD25 deficiency, Stat5b deficiency, and cartilage hair dysplasia (Al-Herz et al., 2011).

Most of these immunodeficiencies are associated with (i) disturbed T cell homeostasis, (ii) altered antigen receptor, or (iii) altered cytokine signaling. Aspects relevant in patients with CVID shall be discussed in the following sections.

DISTURBED T CELL HOMEOSTASIS IN AI-CVID

Disturbed T cell homeostasis is a common contributing factor to the development of autoimmunity in different forms of monogenic primary immunodeficiency disorders (PIDs). Several features of disturbed cell homeostasis are also present in CVID. Lymphopenia affects mostly CD4 T cells and especially naïve CD4 T cells, while CD8 T cells become relatively expanded (Giovannetti et al., 2007). Both CD4 and CD8 T cells are activated as determined by the expression of activation markers and Ki67. Thymic output was decreased, but Ki67 expression was particularly strong in naïve and central memory T cells, suggesting homeostatic

proliferation as described for other immunodeficiency models (Cassani et al., 2010). In addition, the Vβ repertoire of CD4 T cells had contracted. These changes are well known to be associated with an increased risk of autoimmunity as previously demonstrated in murine models and human AI disease (Datta and Sarvetnick, 2009).

The severe reduction in naïve CD4 T cells in CVID has been suggested as a criterion for the diagnosis of late-onset CID (LOCID; Malphettes et al., 2009) for resembling the immunological and clinical phenotype of patients with hypomorphic SCID mutations (Liston et al., 2008; Cassani et al., 2010; De Ravin et al., 2010). Interestingly, the association of CD4 lymphopenia in primary immunodeficiency seems to be stronger with granulomatous inflammatory disease than AI cytopenias (Schuetz et al., 2008; Mouillot et al., 2010). IL-7, which has a key role in the expansion of autoreactive T cell clones in the lymphopenic host, was also found to be elevated in a subgroup of CVID patients (Holm et al., 2005). Though increased IL-7 levels were not associated with T cell lymphopenia, they nevertheless correlated with a more frequent incidence of autoimmunity. The regular feedback mechanism of IL-7 regulation seemed to fail in the small group of AI-CVID patients examined. The production of several other cytokines including IL-2, interferon (IFN)-γ, IL-4, and TNFα is altered in some CVID patients, but none of the reported alterations have been examined for their role in eliciting autoimmunity (Fischer et al., 1994; Fritsch et al., 1994; Mullighan et al., 1997). Testing the role of specific cytokines in this setting will be of great interest as it is likely to reveal potential therapeutic targets.

Skewing of CD8 T cells is often more prominent than that of CD4 T cells (Giovannetti et al., 2007). Cytomegalovirus (CMV) causes immunosenescence associated with terminal differentiation of CD8 effector T cells which results in a skewing of the repertoire. In CVID this phenomenon was exaggerated (Kuntz et al., 2011). A chronic viral infection is therefore a potential trigger for the clinical manifestation of AI disease in a disturbed immune system (Marashi et al., 2011).

Selection, activation, and differentiation of T cells in CVID may also be affected by an impaired response of the T cell receptor after stimulation (Fischer et al., 1994; Boncristiano et al., 2000; Paccani et al., 2005). However, to date, the published investigations neither report an underlying genetic defect nor a correlation between altered T cell receptor signaling and a higher prevalence of autoimmunity. Currently, the only intrinsic T cell defect which causes CVID was found in a total of 11 patients with deficiency of the inducible costimulator (ICOS; Warnatz et al., 2006; Takahashi et al., 2009). Only one of the original nine European patients presented with AI neutropenia, whereas AI manifestations were more prominent in the two Japanese patients presenting with (rheumatoid) arthritis, inflammatory bowel disease, interstitial pneumonitis, and psoriasis.

Finally, many reports have described reduced numbers of circulating regulatory T cells in CVID, especially affecting Freiburg Ia patients with reduced switched memory B cells and expansion of $CD21^{low}$ B cells (see below; Fevang et al., 2007; Genre et al., 2009; Horn et al., 2009; Melo et al., 2009; Yu et al., 2009; Arumugakani et al., 2010; Mouillot et al., 2010). Several of the factors mentioned above, such as a CID-like phenotype with or without a disturbed TCR signal (Picard et al., 2009; Sauer et al., 2012), cytokine disturbance (Setoguchi et al., 2005), and even persistent CD4 lymphopenia itself (Matsuoka et al., 2010) might contribute to the reduction in regulatory T cells. Interestingly, even ICOS deficiency disturbs maintenance and function of regulatory T cells (Kornete et al., 2012), thus potentially rendering regulatory T cell deficiency a crucial element in AI dysregulation which is also common to different forms of immunodeficiency.

DISTURBED B CELL HOMEOSTASIS IN AI-CVID

B cell homeostasis is also disturbed in CVID patients. Therefore, reduced switched memory B cell development and the expansion of activated $CD21^{low}$ B cells are associated with the manifestation of AI-CVID (Warnatz et al., 2002; Sanchez-Ramon et al., 2008; Isnardi et al., 2010; Boileau et al., 2011). $CD21^{low}$ B cells contain a high proportion of autoreactive clones (Rakhmanov et al., 2009; Isnardi et al., 2010) suggesting a disturbed selection of the B cell repertoire. This may involve defects in central selection for some (Isnardi et al., 2010), but not all patients (Rakhmanov et al., 2010). Several factors have been identified as interfering with B cell selection. Firstly, the signal strength of the BCR itself determines the outcome during selection (Khan, 2009). Several mouse models have demonstrated that alterations in the signaling machinery (Cornall and Goodnow, 1998; Wang and Clark, 2003) and the balance between co-stimulatory (Tedder et al., 1997) and inhibitory co-receptors (Cornall et al., 1998) determine the counter-selection of AI B cell clones. In CVID patients disturbed antigen receptor signaling was described and is discussed below.

Given the negative feedback loop of immune complexes on B cells and plasma cells via the inhibitory receptors (Seite et al., 2010; Baerenwaldt et al., 2011) it is intriguing to speculate as to whether low serum IgG by itself may contribute to antibody-mediated AI cytopenias as one of the first manifestations in AI-CVID. Signaling by FcγRIIB inhibits B cell activation and can even induce apoptosis in plasma cells (Xiang et al., 2007). Additionally, a lack of inhibition of monocytes/macrophages by FcγRIIB may foster overwhelming inflammatory responses and granuloma formation, a serious clinical problem seen in a subset of AI-CVID patients. Lupus-like disease in FcγRIIB-deficient C57BL/6 mice (Bolland and Ravetch, 2000) as well as the increased risk of SLE in homozygous carriers of the dysfunctional FcγRIIB I232T variant (Floto et al., 2005) clearly indicate a crucial role for this inhibitory receptor in the maintenance of humoral tolerance. This hypothesis is supported by the fact that in most CVID patients the initiation of immunoglobulin replacement leads to an amelioration of the bouts of AI-mediated cytopenias.

The other major factors, which contribute to B cell-mediated autoimmunity, are related to survival signals during selection (Cancro, 2004). For B cells, overexpression of B cell-activating factor (BAFF) causes increased survival of autoreactive B cells and overt autoimmunity (Mackay et al., 1999; Thien et al., 2004). It is noteworthy that most CVID patients present with elevated BAFF levels (Kreuzaler et al., 2012). Currently it is unknown whether elevated BAFF levels sustain the expansion of $CD21^{low}$ B cells in

CVID. The number of circulating CD21^{low} B cells increases in other AI diseases, such as SLE (Wehr et al., 2004), rheumatoid arthritis (Isnardi et al., 2010), and cryoglobulinemia (Terrier et al., 2011), supporting an association with autoimmunity. In contrast to SLE, where switched memory B cells are relatively expanded and active disease is associated with expansion of circulating plasmablasts (Dorner and Lipsky, 2004), AI-CVID has a more severe reduction in the number of switched memory B cells when compared to other CVID patients. This could represent a disturbed peripheral differentiation and selection. Increased autoimmunity associated with poor germinal center function has also been observed in deficiency of the AID (Hase et al., 2008), but no abnormalities of AID expression or function have been described in CVID at this point.

Of all the genetic mutations which are associated with CVID, AI manifestations are most common in TACI-deficiency [18/50 (36%) vs 112/490 (23%) in wt TACI CVID; Salzer et al., 2009]. In particular, heterozygous C104R mutations seem to effect a predisposition for autoimmunity (11/20 patients, 55%; Salzer et al., 2009). While partial TACI signals in a heterozygous state may contribute to the survival of autoreactive B cells, a formal proof of this hypothesis is still missing. AI manifestations including glomerulonephritis and vasculitis (interestingly with deposits of IgA) as well as AI thrombocytopenia (AI-TP) have also been described for CD19 and CD81 deficiency, and are possibly related to the disturbed antigen receptor signal in these patients (see also below; van Zelm et al., 2006, 2010; Vince et al., 2011). The other B cell-intrinsic genetic defects associated with CVID (BAFF-R, CD20, CD21) have not been reported with AI manifestations (Warnatz et al., 2009; Kuijpers et al., 2010; Thiel et al., 2011, but to date only single patients have been described for each defect, thus precluding definite conclusions.

In recent years, a B cell population producing IL10 has been described as regulatory B cells (Mauri and Bosma, 2012). Currently, nothing is known about their existence and function in CVID.

DISTURBED ANTIGEN RECEPTOR SIGNAL IN AUTOIMMUNE CVID

Several mouse models of increased BCR signals demonstrate an increased prevalence of AI manifestations (Dorner and Lipsky, 2006). On the other hand, models of decreased TCR signaling can also represent a risk factor for autoimmunity (summarized in Liston et al., 2008). Decreased TCR signals are thought to interfere with negative selection either through a selective or a stronger impact on tolerogenic signals (Liston et al., 2008) thus potentially impairing the generation of regulatory T cells (Liston and Rudensky, 2007). In humans, ORAI (Feske et al., 2006) and Stim1 deficiency (Picard et al., 2009) need to be mentioned as prototypes of reduced antigen receptor signal strongly associated with the coincidence of immunodeficiency and autoimmunity in the affected patients. Also in B cells of the subgroup of CVID patients with an increased risk of AI manifestations, calcium signaling is reduced compared to other CVID patients and healthy controls (Foerster et al., 2010; van de Ven et al., 2011). The exact mechanism of the signaling defect and its potential interference with selection are unknown. In WAS, antigen receptor signaling is impaired due to mutations in the WAS protein (Zhang et al., 1999). Interestingly, WASP deficiency also leads to increased AI disease associated with decreased CD27^{+} memory B cells and increased CD21^{low} B cells (Park et al., 2005). Although WASP deficiency affects both T and B cell receptor signaling, B cell-intrinsic defects clearly contribute to autoimmunity in WAS (Recher et al., 2012). As indicated above, previous reports have found disturbed TCR-induced calcium signals (Fischer et al., 1996) in 40–50% of CVID patients but a link to immune dysregulation in the identified patients has not been established.

ALTERED TYPE I INTERFERON SIGNAL IN AUTOIMMUNE CVID

Cytokines have been implicated in AI dysregulation. Type I IFNs are thought to be particularly important as (i) AI reactions are induced in patients after treatment with type I IFNs, (ii) the *IFN* signature is increased in patients with SLE, and (iii) some chronic viral infections are associated with autoimmunity (Hall and Rosen, 2010). The mechanisms are manifold and include induction of dendritic cell (DC) maturation and increased BAFF production, a positive feed back loop in toll-like-receptors (TLR) 7 and 9 signaling leading to class switched antibody production (Hall and Rosen, 2010).

Type I IFNs have not been well examined in CVID patients. There exists only a single report of increased type I IFN production in CVID patients (Strannegard et al., 1987); others have detected increased MxA expression as a marker of IFN exposure in leukocytes of only 2/13 CVID patients (Rump et al., 1995). So far no attempt to correlate in CVID IFN expression to AI manifestations has been made.

Type I IFN expression and the induction of AI reactions is closely linked to the activation of TLRs on plasmacytoid DCs (pDCs) and B cells (Green and Marshak-Rothstein, 2011). Different strains of AI prone mice rendered deficient in TLR7/9 or MyD88 expression produce dramatically fewer autoantibodies and develop less severe disease (Green and Marshak-Rothstein, 2011). Surprisingly, however, TLR9 deficiency in the presence of normal TLR7 function reduces only anti double-strain-DNA autoantibody levels, but not other autoantibodies and is associated with a more severe AI disease, suggesting a regulatory role of TLR9 for TLR7-mediated immune disease. In CVID patients, pDC and B cell responses to TLR7 and 9 ligands are impaired (Yu et al., 2012). Subanalysis of the reported data suggests that a subgroup of patients is more seriously affected by reduced TLR signaling. While the authors correlate the reduced function to increased infection susceptibility no correlation to autoimmunity is mentioned.

In summary, autoimmunity is a prominent clinical feature in CVID. Associated factors include disturbed B and T cell homeostasis and selection, altered antigen receptor signals, increased BAFF levels, and possibly altered TLR signaling. Pathogenic mechanisms, however, have not been identified yet on a molecular level. Further research needs to consider established mechanisms in other genetically defined immunodeficiency disorders to unravel the underlying immune dysregulation in CVID. Our improved knowledge will not only steer potential treatment strategies but also our concept of autoimmunity in general.

ACKNOWLEDGMENTS

This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 620 project C1 (to Klaus Warnatz) and FOR 831 project 8 (VO673/31, to Reinhard E. Voll) and by the German Federal Ministry of Education and Research (BMBF 01 EO0803, to Klaus Warnatz and Reinhard E. Voll). The authors are responsible for the contents of this publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 April 2012; accepted: 01 July 2012; published online: 18 July 2012.
*Citation: Warnatz K and Voll RE (2012) Pathogenesis of autoimmunity in common variable immunodeficiency. Front. Immun. **3**:21. doi: 10.3389/fimmu.2012.00210*
This article was submitted to Frontiers in Primary Immunodeficiencies, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 24 July 2012
doi: 10.3389/fimmu.2012.00189

Autoimmune cytopenias in common variable immunodeficiency

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Common variable immunodeficiency (CVID) is a humoral immunodeficiency whose primary diagnostic features include hypogammaglobulinemia involving two or more immunoglobulin isotypes and impaired functional antibody responses in the majority of patients. While increased susceptibility to respiratory and other infections is a common thread that binds a large cross-section of CVID patients, the presence of autoimmune complications in this immunologically and clinically heterogeneous disorder is recognized in up to two-thirds of patients. Among the autoimmune manifestations reported in CVID (20–50%; Chapel et al., 2008; Cunningham-Rundles, 2008), autoimmune cytopenias are by far the most common occurring variably in 4–20% (Michel et al., 2004; Chapel et al., 2008) of these patients who have some form of autoimmunity. Association of autoimmune cytopenias with granulomatous disease and splenomegaly has been reported. The spectrum of autoimmune cytopenias includes thrombocytopenia, anemia, and neutropenia. While it may seem paradoxical "*prima facie*" that autoimmunity is present in patients with primary immune deficiencies, in reality, it could be considered two sides of the same coin, each reflecting a different but inter-connected facet of immune dysregulation. The expansion of CD21 low B cells in CVID patients with autoimmune cytopenias and other autoimmune features has also been previously reported. It has been demonstrated that this unique subset of B cells is enriched for autoreactive germline antibodies. Further, a correlation has been observed between various B cell subsets, such as class-switched memory B cells and plasmablasts, and autoimmunity in CVID. This review attempts to explore the most recent concepts and highlights, along with treatment of autoimmune hematological manifestations of CVID.

Keywords: common variable immunodeficiency (CVID), autoimmune cytopenias, immune thrombocytopenia, autoimmune hemolytic anemia, autoimmune lymphoproliferative syndrome, Evans syndrome

INTRODUCTION

Common variable immunodeficiency (CVID) is a highly heterogeneous immunodeficiency with varying complexity. The key diagnostic elements include low IgG (2 SD below mean of age) along with low IgA and/or IgM (Park et al., 2008; Resnick et al., 2011). CVID is considered the most commonly encountered and clinically relevant primary immunodeficiency in adults (Chapel et al., 2008; Park et al., 2008) and though the majority of patients are diagnosed between the age of 20 and 40 years, at least another 20% are diagnosed during childhood (>2 years) or adolescence (Cunningham-Rundles, 2010).

While recurrent sinopulmonary infections are one of the hallmarks of this disease, gastrointestinal, viral, and systemic bacterial infections have also been reported (Park et al., 2008; Resnick et al., 2011). Besides infections, CVID is associated with a variety of non-infectious complications including pulmonary disease, autoimmunity, granulomatous disease, gastrointestinal disease, and malignancy (Chapel et al., 2008; Resnick et al., 2011).

The clinical heterogeneity and complexity of CVID has led to renewed efforts over the past decade to identify causal genetic defects as well as correlate the "immuno-phenotype" with clinical phenotype (Warnatz et al., 2002; Piqueras et al., 2003; Wehr et al., 2008; Eibel et al., 2010). In the last 10 years, monogenic defects associated with antibody deficiency have been described in a small subset of CVID patients or patients with hypogammaglobulinemia, or single or few families with a history of consanguinity. These genetic defects include disease-causing mutations or polymorphisms in the *TNFRSF13B* (TACI), *CD19*, *ICOS*, *TNFRSF13C* (*BAFF-R*), *CD81*, *CD20*, *MSH5*, and *CD21* genes (Grimbacher et al., 2003; Salzer et al., 2004, 2005, 2009; Castigli et al., 2005, 2007; Warnatz et al., 2005; van Zelm et al., 2006, 2010; Kanegane et al., 2007; Pan-Hammarstrom et al., 2007; Schaffer et al., 2007; Sekine et al., 2007; Zhang et al., 2007; Kuijpers et al., 2010; Frank, 2012; Thiel et al., 2012). However, single-gene defects were identified in only a relatively small subset of CVID patients raising the possibility that the majority (>75%) of CVID patients have oligogenic or polygenic defects. This was recently substantiated by a genome-wide association study of 363 CVID patients, which revealed that copy number variations (CNV), including gene duplications and/or deletions were present and this analysis led to the identification of several "novel" genes, which may play an important role in the immune response, and genetic variations

therein could lead to a disease phenotype associated with CVID (Orange et al., 2011).

Paradoxical as it may seem, autoimmune manifestations are not uncommon in patients with primary immunodeficiencies (PIDDs) and at least 25% of all PIDDs described in the 2011 IUIS classification may have some form of autoimmune phenomenon (Bussone and Mouthon, 2009; Notarangelo, 2009; Al-Herz et al., 2011). The autoimmunity observed in PIDDs may be related either to a direct or indirect genetic effect, and includes defects in genes that regulate immunological self-tolerance as well as genetic variations that alter immune regulation. Not surprisingly, therefore, autoimmune features are identified relatively frequently in CVID patients (Brandt and Gershwin, 2006; Knight and Cunningham-Rundles, 2006; Cunningham-Rundles, 2008).

AUTOIMMUNITY IN CVID

Autoimmune hematological abnormalities, specifically cytopenias, are the most common of all autoimmune manifestations in CVID and may present as thrombocytopenia, anemia or neutropenia. In the longitudinal study mentioned above, immune thrombocytopenia (ITP) was reported in 14% of patients, while autoimmune hemolytic anemia (AIHA) and neutropenia was less common with only 7 and <1%, respectively, of the cohort affected (Resnick et al., 2011). It should also be kept in mind that autoimmune cytopenias may in fact be the presenting symptom for a small subset of CVID patients, especially in children, where Evans syndrome (ES) has been reported to precede the clinical and immunological phenotype of CVID (Savasan et al., 2007). Other autoimmune presentations reported in CVID include rheumatoid arthritis, anti-IgA antibodies, vitiligo, and alopecia (Horn et al., 2007; Park et al., 2008; Resnick et al., 2011). A very recent longitudinal study assessing clinical complications that cause morbidity and mortality in CVID patients identified autoimmune complications in 29% of a cohort of 473 patients studied over 4 decades (Resnick et al., 2011). Interestingly, in the same study, the presence of autoimmunity was not associated with an increase in mortality.

IMMUNOLOGICAL AND PHENOTYPIC MANIFESTATIONS OF AUTOIMMUNE CYTOPENIAS IN CVID

As alluded to previously, several clinical and immunological classifications have been posited in an attempt to stratify and may be even simplify the complex and heterogeneous phenotypes seen in CVID (Warnatz et al., 2002; Piqueras et al., 2003; Chapel et al., 2008; Wehr et al., 2008). The relatively more recent EUROclass study attempted to cohesively link the earlier Freiburg and Paris classifications by correlating B cell subset immunophenotypes with clinical presentation specifically providing correlation for autoimmunity, granulomatous disease, and splenomegaly (Warnatz et al., 2002; Piqueras et al., 2003; Wehr et al., 2008). Of particular relevance was the correlation of an expansion of $CD21^{low/dim}$ B cells with splenomegaly (Wehr et al., 2008). The $CD21^{low/dim}$ B cells have been previously reported to be a subset of anergic B cells with defective signaling that has the capacity to home to sites of inflammation (Rakhmanov et al., 2009, 2010; Foerster et al., 2010; Charles et al., 2011). Additionally, correlations were identified between an expansion of transitional B cells with lymphadenopathy and autoimmune cytopenias with reduced plasmablasts – pre-terminally differentiated plasma cells (Wehr et al., 2008).

Data from Sanchez-Ramon et al. (2008) and Vodjgani et al. (2007) provide independent substantiation of the association between low class-switched memory B cells and clinical features of autoimmunity and splenomegaly in CVID patients reported by the EUROclass and other classification studies (Warnatz et al., 2002; Piqueras et al., 2003; Wehr et al., 2008).

Martinez-Gamboa et al. (2009) showed that there was a numerical decrease in memory B cell numbers in ITP patients who underwent splenectomy and alluded to a potential role for the spleen in maintaining memory B cell homeostasis. However, a different study suggests that the age at which splenectomy is performed is more relevant to maintenance of marginal zone (memory) B cells numbers than consideration of splenectomy in isolation, regardless of age at which the procedure is done (Wasserstrom et al., 2008).

Besides the correlation of B cell subsets, specifically switched memory B cells, with autoimmunity, there is evidence from multiple human and mouse models on the significance and importance of regulatory T cells expressing FOXP3 in suppressing or controlling autoimmunity (Buckner, 2010; Long and Buckner, 2011). It has been shown in at least a subset of CVID patients, particularly those with autoimmune features, that there is a substantial decrease in relative frequency (%) but not absolute quantitation of FOXP3+ Tregs raising the possibility of abnormal immune regulation in these patients (Arumugakani et al., 2010), though the mechanism of immune dysregulation in this context may extend beyond numerical changes to possible functional alterations as well (Jang et al., 2011; Long and Buckner, 2011).

Another recent study demonstrated B cell receptor recombination bias in a subset of CVID patients and postulated that this may predispose to decreased secondary recombination with subsequent defective central tolerance leading ultimately to the escape of autoreactive clones (Romberg et al., 2011). Further, a biomarker (soluble BAFF/BLys) produced by monocytes and dendritic cells (DCs), which is a critical B cell survival and proliferation factor, and known to be abnormally increased in contexts of autoimmunity, especially in rheumatologic diseases (Becker-Merok et al., 2006) was also been shown to be elevated in CVID patients but there was no demonstrable correlation with the incidence of autoimmunity (Knight et al., 2007).

CVID: OVERLAP WITH AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME AND EVANS SYNDROME

Published data have demonstrated a clear immunologic and clinical overlap between CVID, ES, and autoimmune lymphoproliferative syndrome (ALPS). ES is characterized by the presence of autoimmune cytopenias in two or more hematopoietic lineages. A small study evaluating 12 pediatric patients with ES determined that half (6/12) also had elevated αβ TCR+ DNT T cells (CD3+CD4–8–) and defective Fas apoptosis characteristic of ALPS patients (Teachey et al., 2005). A subsequent larger study of 45 patients with ES substantiated the earlier finding

by demonstrating diagnostic criteria for ALPS in 21/45 patients (Seif et al., 2010).

The correlation between ES, ALPS, and CVID was made in a different study, which though limited in sample size ($n = 7$), showed development of hypogammaglobulinemia, as seen in CVID in 5/7 patients with ES. These patients also had increased Fas expression (Savasan et al., 2007). A larger cohort study of 68 patients with ES showed that only a relatively small proportion, 4/68 had CVID (Michel et al., 2009).

In a separate study of ALPS patients ($n = 66$), an equally small number, 5/66 had hypogammaglobulinemia, suggesting a potential phenotypic overlap with CVID. The majority of the ALPS patients in this study had reduced class-switched memory B cells, similar to what has been reported in two-third or greater of CVID patients (Rensing-Ehl et al., 2010).

MECHANISMS OF DEVELOPMENT OF AUTOREACTIVITY

The development of self-reactive B cells is regulated both centrally (bone marrow) and peripherally through at least two independent check-points. It has been suggested that there may be a failure of both central and peripheral tolerance mechanisms in CVID due to immune dysregulation resulting in a flawed negative selection process. Logically, this would suggest that there would be an increased selection of autoreactive B cells prior to affinity maturation (somatic hypermutation) or memory B cell/plasma cell commitment in the secondary lymphoid organs (Haymore et al., 2008). This is a topic that is discussed in depth elsewhere in this journal series, and therefore, not addressed herein.

DIAGNOSIS AND TREATMENT

DIAGNOSIS

The evaluation of CVID patients for autoimmune cytopenias should include appropriate diagnostic work-up (**Figure 1**), however, in the case of ITP this may primarily be a diagnosis of exclusion. A presumptive diagnosis of ITP can be arrived at by ruling out alternative pathological mechanisms through clinical history, physical review, complete blood count (CBC) analysis, and peripheral blood smears (Provan et al., 2010). Confirmation of the diagnosis is usually determined by response to appropriate treatment. As per the previous discussion that autoimmune cytopenias may precede a diagnosis of CVID, it would be reasonable to evaluate both pediatric and adult patients for immunoglobulin levels on diagnosing ITP to rule out a possible CVID or selective IgA deficiency (Provan et al., 2010). Additionally, follow-up may be required with periodic evaluation and correlation with clinical history to document evolution of the disease process.

Likewise, the diagnosis of AIHA mandates evidence of hemolysis along with detection of an autoantibody. There are a number of laboratory markers for establishing hemolysis, including a CBC with peripheral smear, increased indirect bilirubin, increased lactate dehydrogenase (LDH), and decreased haptoglobin. Autoantibodies can be detected by a direct antiglobulin test (DAT) or Coomb's test (Gehrs and Friedberg, 2002).

The diagnosis of autoimmune neutropenia (AIN) is similar to ITP in that it is a diagnosis of exclusion. In some cases, detection of anti-granulocyte antibodies may be useful but the lack of detectable autoantibodies does not exclude a diagnosis of AIN (Bope and Kellerman, 2012). Most cases of AIN are associated with normal marrow reserve and pathogenesis is related to antibody-mediated destruction and in some cases, sequestration. The diagnosis can include a bone marrow biopsy, which would reveal a hypercellular marrow and usually a late maturational arrest, though in some cases, an early arrest can also be seen. AIN may be associated with ITP and/or AIHA in CVID patients. Besides, the possible presence of anti-neutrophil antibodies, circulating immune complexes may also be present in a subset of patients with AIN (Dinauer and Coates, 2009).

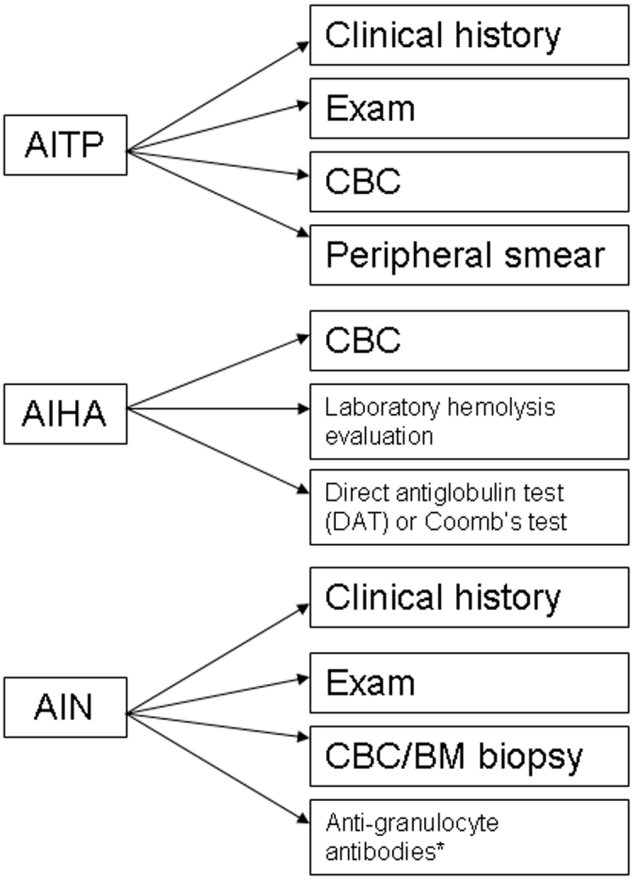

FIGURE 1 | Diagnostic algorithm for autoimmune cytopenias in CVID. AITP, autoimmune thrombocytopenia purpura; AIHA, autoimmune hemolytic anemia; AIN, autoneutropenia; IVIG, intravenous immunoglobulin;. *non-exclusionary.

TREATMENT

A treatment algorithm for autoimmune cytopenias in CVID is provided in **Figure 2**. The American Society of Hematology has provided guidelines for the treatment of patients with ITP and these include initiation of treatment in adult patients if the platelets are below 30×10^9/L. However, in pediatric patients, the current guidelines state that treatment is based on clinical symptoms associated with thrombocytopenia regardless of the platelet counts (Neunert et al., 2011). Pediatric patients are far

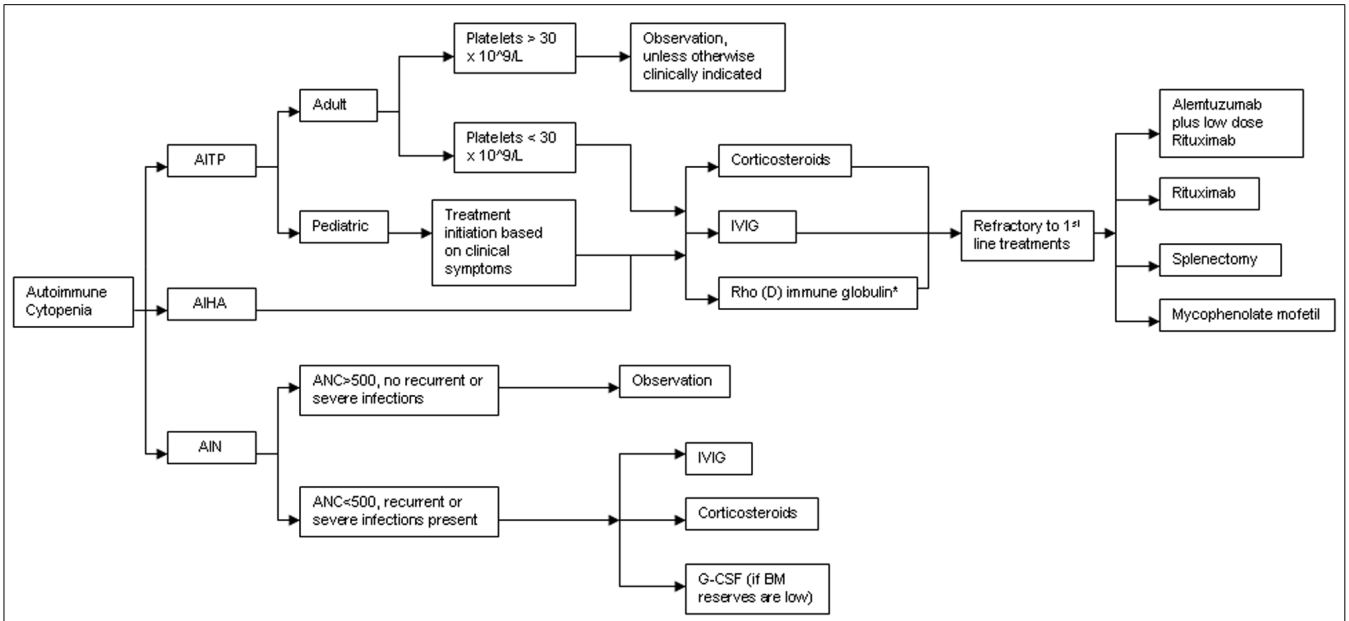

FIGURE 2 | Treatment algorithm for autoimmune cytopenias in CVID. AITP, autoimmune thrombocytopenia; AIHA, autoimmune hemolytic anemia; AIN, autoneutropenia; IVIG, intravenous immunoglobulin; ANC, absolute neutrophil count; G-CSF, granulocyte colony stimulating factor. *Rh+, non-splenectomized individuals only.

more likely to experience spontaneous remissions. The treatment of choice as first-line therapy for ITP is the use of steroids at 1 mg/kg for a duration of at least three weeks with subsequent dose reduction and eventual withdrawal. Alternative therapeutic options could include a single dose of intravenous immunoglobulin (IVIG) at 1 g/kg. Further use of IVIG is dependent on clinical response to the initial dose. A combination of the above two therapies may be utilized if a rapid response is required. Rho(D) immune globulin is an option for Rh-positive individuals who have not undergone a splenectomy and are unable to tolerate steroid treatment (Neunert et al., 2011). Splenectomy is recommended as a therapeutic option only for those patients that fail corticosteroid therapy. CVID patients undergoing splenectomy or receiving immunosuppressive medication may be at increased risk for infection given their intrinsic immunological defects.

While AIHA is treated much like ITP, it may be more challenging to manage, particularly in patients with ES (Cunningham-Rundles, 2002; Wang and Cunningham-Rundles, 2005). For refractory cases of ITP, AIHA, or both, Rituximab, a chimeric monoclonal anti-CD20 B cell-depleting agent, has been effectively used. In a modest-size cohort of CVID patients ($n = 33$) with refractory autoimmune cytopenias (failure of at least 2–6 treatments prior to initiation of Rituximab), the initial response rate was remarkably high at 84% (Gobert et al., 2011). Severe infection was an unfortunate consequence in almost a quarter of these patients (8/33) over a mean follow-up period of 39 months. Of note, half the patients (4/8) were not on replacement immunoglobulin therapy at the time of infectious diagnosis. An earlier study reports similar rates of infection in patients with ITP who received standard treatment (Michel et al., 2004).

The treatment of AIN is primarily dictated by the severity of neutropenia-associated clinical symptoms and the underlying disease context. Treatment with high-dose IVIG or steroids may be used if there is very profound neutropenia (ANC $< 500/mm^3$) in conjunction with recurrent or fulminant infections. G-CSF therapy is only of value if bone marrow reserves are depleted. Splenectomy has little value in reversing neutropenia, especially if it is isolated, since the effect is transient, and can ultimately increase overall infection risk (Dinauer and Coates, 2009).

A separate study of 19 adult patients with steroid-refractory autoimmune cytopenias, reported a 100% initial response rate to a combination of low-dose Rituximab and Alemtuzumab (anti-CD52 humanized monoclonal antibody). Infection occurred in 6/19 patients after a median period of 70 weeks (Gomez-Almaguer et al., 2010). Other reports have documented an initial response rate of 78–92% for refractory autoimmune cytopenias treated with mycophenolate mofetil with no significant adverse events reported (Kotb et al., 2005; Rao et al., 2005). Thus, the approach to treating autoimmune cytopenias in CVID is not dissimilar to the treatment of immune competent patients (Wang and Cunningham-Rundles, 2005).

SUMMARY

This minireview, which is limited in scope, provides an encapsulated discussion on the incidence and presentation of autoimmunity in CVID, specifically autoimmune cytopenias, their overlap with other clinical entities, some notable immunological hallmarks, laboratory diagnosis and an overview of standard and new therapies. As mentioned in the text, a more exhaustive treatment of autoimmunity in CVID, focusing on mechanistic aspects, is provided elsewhere.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 February 2012; accepted: 18 June 2012; published online: 24 July 2012.
*Citation: Podjasek JC and Abraham RS (2012) Autoimmune cytopenias in common variable immunodeficiency. Front. Immun. **3**:189. doi: 10.3389/fimmu.2012.00189*
This article was submitted to Frontiers in Primary Immunodeficiencies, a specialty of Frontiers in Immunology.

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IMMUNOLOGY

MINI REVIEW ARTICLE
published: 04 June 2012
doi: 10.3389/fimmu.2012.00129

TH17 cells in autoimmunity and immunodeficiency: protective or pathogenic?

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In 2005 a newly discovered T helper cell subset that secreted interleukin (IL)-17 became the center of attention in immunology. Initial studies painted Th17 cells as the culprit for destruction in many different autoimmune and auto-inflammatory diseases. Subsequently, the discovery of patients with primary immunodeficiencies in the IL-17 pathway taught us that Th17 cells have a critical role in defense against certain fungal and bacterial infections. Moreover, the paradoxical exacerbation of Crohn's disease in the clinical trials of a Secukinumab (AIN457), a fully human neutralizing antibody to IL-17A, has cast into doubt a universal pro-inflammatory and harmful role for Th17 cells. Evidence now suggests that depending on the environment Th17 cells can alter their differentiation program, ultimately giving rise to either protective or pro-inflammatory cells. In this review we will summarize the evidence from patients with immunodeficiencies, autoimmune, or auto-inflammatory diseases that teaches us how the pro-inflammatory versus protective function of Th17 cells varies within the context of different human diseases.

Keywords: Th17 cells, autoimmunity, T regulatory cells, immunodeficiency, inflammatory bowel disease, psoriasis, type 1 diabetes, secukinumab

INTRODUCTION

In rare cases, a mutation in an essential gene can disrupt immune homeostasis, leading to clinical immunodeficiency. More commonly, when individuals with a genetic predisposition are exposed to environmental triggers, a failure of immune homeostasis can lead to autoimmunity. In this review, we will discuss how parallel studies of immunodeficiencies and autoimmune diseases have advanced our knowledge of a $CD4^+$ T cell lineage first characterized by production of IL-17A, Th17 cells.

Th17 cells were identified in 2005 (Harrington et al., 2005; Langrish et al., 2005; Park et al., 2005) and, as for other $CD4^+$ T cell lineages, their development, is controlled by a combination of cytokines which initiate a program of transcription factor expression and epigenetic re-modeling (van der Gast et al., 2011). In humans, the cytokines which instruct Th17 cell lineage development likely include IL-6, IL-21, IL-23, and IL-1β (Acosta-Rodriguez et al., 2007a; Chen et al., 2007; Evans et al., 2007; Wilson et al., 2007; Liu and Rohowsky-Kochan, 2008), with a potential synergistic role for TGF-β (Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008a) via its ability to suppress Th1 cell lineage commitment (Santarlasci et al., 2009). Cytokine-driven activation of the signal transducer and activator of transcription (STAT) 3 pathway is an essential step in Th17 cell differentiation (Holland et al., 2007; Yang et al., 2007; Ma et al., 2008), ultimately leading to expression of their lineage-defining transcription factor: retinoid orphan receptor (ROR)C2 (Acosta-Rodriguez et al., 2007a; Annunziato et al., 2007; Wilson et al., 2007; Manel et al., 2008; Crome et al., 2009). Although the IL-17 cytokine family includes six members (Kolls and Linden, 2004), Th17 cells are thought to only produce IL-17A and IL-17F, which are 55% identical (Kolls and Linden, 2004). IL-17A can combine with IL-17F to form a heterodimer and both can form homodimers (Wright et al., 2007).

Th17 cells have many phenotypic characteristics that distinguish them from other Th cell lineages. In addition to IL-17A and IL-17F, Th17 cells secrete other signature cytokines including IL-21 and IL-22 (Bending et al., 2011). They have also been reported to produce IFN-γ (Annunziato et al., 2007), IL-4 (Cosmi et al., 2010), IL-10 (McGeachy et al., 2007), IL-9 (Beriou et al., 2010), IL-26, CXCL8, and CCL20 (Boniface et al., 2008). They are poor producers of IL-2, which may result in their poor proliferative potential *in vitro* (Santarlasci et al., 2012). They constitutively co-express CCR4 and CCR6, but not CXCR3 (Acosta-Rodriguez et al., 2007b), and are derived from $CD161^+$ precursors (Cosmi et al., 2008). The effects of Th17 cells on other cells have recently been highlighted in many reviews (Annunziato and Romagnani, 2011; Gaffen, 2011; Gaffen et al., 2011; Ghoreschi et al., 2011; Milner, 2011; Pappu et al., 2011; Wilke et al., 2011).

Th17 cells initially developed a reputation as a destructive element in several diseases including multiple sclerosis (MS), rheumatoid arthritis (RA), psoriasis, and inflammatory bowel disease (IBD). In animal models this reputation, came from evidence that the lack of IL-17-producing cells ameliorates experimental autoimmune encephalitis (EAE) and collagen induced arthritis (CIA; Cua et al., 2003). In humans, the reputation was due to correlative data documenting an increase in IL-17-producing cells, particularly at sites of tissue inflammation (Wilke et al., 2011). However, these original conclusions were over-simplified

and as discussed below in some diseases Th17 cells clearly have a protective role.

THE ROLE OF Th17 CELLS IN PRIMARY IMMUNODEFICIENCIES

Much of what we know about human Th17 cells comes from the study of a rare primary immunodeficiency called Hyper-immunoglobulin E (Job's, syndrome). This disease is caused by mutations in *STAT3* (Holland et al., 2007) but the underlying cellular basis for the characteristic phenotype of severe pneumonias, mucocutaneous candidiasis, and *Staphylococcus aureus* abscesses (Buckley et al., 1972; Grimbacher et al., 1999) remained unknown until several groups found that these patients lack Th17 cells in their peripheral blood (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008). In addition, naïve Th cells from Job's syndrome patients have low levels of RORC2 expression and cannot be differentiated into Th17 cells *in vitro* (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008).

One complication when interpreting data from Job's syndrome patients is that STAT3 is activated downstream of other cytokines, making it difficult to attribute a clinical phenotype to one pathway. Recently, other immunodeficiencies have been described which involve more specific defects in the IL-17 pathway. For example, two patients with chronic mucocutaneous candidiasis (CMC) disease, characterized by chronic or persistent infection with *Candida albicans* and *S. aureus*, were found to have an IL-17RA autosomal recessive deficiency or an IL-17F autosomal dominant deficiency (Puel et al., 2011). In addition, patients with a deficiency in the intracellular adaptor molecule CARD9, which is essential for dectin-1 signaling, also suffer from systemic *Candidiasis* infection (Glocker et al., 2009) and have low numbers of Th17 cells in their peripheral blood.

Together, these data have led to the hypothesis that in humans Th17 cells have an essential role in protective immunity the specific pathogens *C. albicans* and *S. aureus*. In accordance with this conclusion, Sallusto et al. have characterized different subsets of human Th17 cells that can be differentiated *in vitro* with antigen specific stimulation by *C. albicans* and *S. aureus* (Zielinski et al., 2012).

Th17 cells have an intriguing close developmental link with $FOXP3^+CD4^+$ regulatory T cells (Tregs). FOXP3 and RORC2 can directly interact via a DNA-independent mechanism, and during Th17 cell development FOXP3 is transiently expressed (Zhou et al., 2008). Moreover, upon activation fully differentiated human Th17 cells preferentially express FOXP3 in comparison to Th1 cells (McMurchy and Levings, unpublished data). Indeed there is increasing evidence for the existence of cells that co-express IL-17 and FOXP3 (Ayyoub et al., 2009; Beriou et al., 2009; Miyara et al., 2009; Voo et al., 2009; Kryczek et al., 2011; Ye et al., 2011).

Immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX) is a triad of autoimmune syndromes including enteropathy, type 1 diabetes, and hyper-IgE (McMurchy et al., 2010), resulting from mutations in *FOXP3*. The cellular basis for this disease has been attributed to Treg dysfunction (Bacchetta et al., 2006; D'Hennezel et al., 2009), but recently Bacchetta et al. investigated whether part of the cellular defect in IPEX patients may not only relate to Treg dysfunction, but also to changes in Th17 cells. Indeed IPEX patients possessed an increased frequency of cells with a surface profile characteristic of Th17 cells ($CD4^+CCR6^+CD161^+$), expressing RORC2, and producing IL-17 (Passerini et al., 2011). Interestingly, in patients with point mutations that do not abrogate FOXP3 expression, there was an increased frequency of $FOXP3^+$ cells within the $CD161^+$ Th17 cell gate. These data suggest that dysfunctional Tregs may preferentially differentiate into Th17 cells and that an expansion of this subset may underlie some of the clinical phenotype of IPEX. An alternative interpretation is that the Th17 cells in IPEX patients are highly activated and that in this case FOXP3 expression is a consequence of T cell activation and not Treg lineage commitment (Ziegler, 2006). Regardless, in this immunodeficiency there is strong correlative evidence that Th17 cells may have a detrimental pro-inflammatory effect.

EVIDENCE FOR THE PRO-INFLAMMATORY ROLE OF Th17 IN HUMAN AUTOIMMUNITY

The first recognition of the importance of Th17 cells came from studies of EAE. The notion of EAE as a Th1-mediated disease was challenged when mice deficient in the p40 subunit of IL-12 were found to be resistant to EAE whereas mice deficient in the p35 subunit were actually more susceptible to disease (Becher et al., 2002). Cua et al. (2003) solved this paradox by using genetically deficient mice to show that IL-23p19 and IL-12p40, but not IL-12p35, were essential for EAE development. IL-23, which shares the IL-12p40 subunit with IL-12, was subsequently found to stabilize Th17 cells, and these cells were found to be the main contributing factor in EAE (Langrish et al., 2005). Subsequently, a correlation between Th17 cells and human autoimmunity was sought. Below we discuss the evidence for a pro-inflammatory role in autoimmunity and describe attempts to target this axis therapeutically.

Psoriasis is an auto-inflammatory skin disease characterized by recurrent demarcated red and scaly skin plaques. These plaques include infiltrating T cells (mainly Th cells) and dendritic cells in the dermis as well as cytotoxic T cells and neutrophil in the epidermis (Lowes et al., 2007). The resulting inflammatory process results in rapid keratinocyte proliferation, abnormal keratinocyte differentiation, and angiogenesis (Lowes et al., 2007). Initially, increased levels of IFN-γ, TNF-α, and IL-12 in the serum and lesions of psoriasis patients labeled this as a Th1-mediated disease (Di Cesare et al., 2009). However, RORC, IL-1β, IL-6, and IL-23 are also increased in psoriatic skin lesions (Di Cesare et al., 2009) leading to the possibility that Th17 and Th1 act in synergy to produce psoriatic inflammation.

Th17 cells are thought to be recruited to the skin by expression of CCL20, the ligand for CCR6, then locally stabilized by IL-1 and IL-23. Since both IL-17 and IFN-γ cause keratinocytes and antigen presenting cells (APCs) to produce more IL-1, IL-23, and CCL20, a positive feedback loop causing keratinocyte proliferation is established (Kryczek et al., 2008; Zaba et al., 2009). Several monoclonal antibodies targeting TNF-α and the p40 subunit shared by IL-12 and IL-23 (Ustekinumab) have been approved for clinical use in psoriasis. Since IL-17 can act synergistically with TNF-α to induce keratinocytes to express inflammatory proteins (Chiricozzi et al., 2011), it is possible that anti-TNF-α acts in part by inhibiting

Th17 cell-driven inflammation. Targeting IL-17 alone with Secukinumab (AIN457) or Ixekizumab, both fully human neutralizing antibodies to IL-17A, is also effective in psoriasis (Hueber et al., 2010; Leonardi et al., 2012), confirming that this is likely a major pathogenic cytokine in this skin disease. Since Th17 cells are not the sole producers of IL-17 [other possible sources of this cytokine in psoriatic plaques include γδ T cells (Cai et al., 2011), mast cells (Lin et al., 2011), neutrophils (Lin et al., 2011), and Tregs (Bovenschen et al., 2011)], whether or not Th17 cells are the major source of this cytokine in skin remains to be determined.

Another disease with strong links to Th17 cells is RA, a chronic autoimmune disease that leads to joint destruction. T cells infiltrating the synovial fluid of RA patients produce high amounts of IL-17A, IL-1β, and IL-6 (Cascao et al., 2010), especially during early disease and pre-treatment (Chabaud et al., 1999; Ziolkowska et al., 2000; Hwang and Kim, 2005; Leipe et al., 2010). Notably, the levels of IL-17 in the synovium correlate with joint damage, whereas those of IFN-γ correlate with protection (Kirkham et al., 2006). Recent evidence supports a role for IL-17F as well as IL-17A in RA, with the two related cytokines acting in synergy to induce other pro-inflammatory cytokines and chemokines in synoviocytes, myeloid cells, and synovial fibroblasts (Lundy et al., 2007; Tran et al., 2007; Hot and Miossec, 2011; van Hamburg et al., 2011). Hence, analogous to the process in psoriasis, a positive pro-inflammatory feedback loop encourages more Th17 differentiation and maintenance in the joint (reviewed in Sarkar and Fox, 2010). Direct clinical evidence for the role of IL-17 in RA comes from recent clinical trials which found that Secukinumab and another anti-IL-17A therapeutic known as LY2439821 significantly benefit these patients (Genovese et al., 2010; Morrison et al., 2011).

Multiple sclerosis is a neurological disease that results from auto-inflammatory damage to the myelin sheaths surrounding nerves in the brain and spinal cord. This disease has historically been associated with the discovery of Th17 cells since, as discussed above, they have a major pathogenic role in EAE (Bettelli et al., 2008; Dong, 2008; Dubin and Kolls, 2008; Weaver and Hatton, 2009). MS patients have increased IL-17 mRNA in their blood as well as cerebrospinal fluid (Matusevicius et al., 1999), and expression of miRNA326 in their peripheral blood mononuclear cells promotes Th17 cell differentiation and correlates with disease severity (Du et al., 2009). Blood-brain barrier endothelial cells layers are more permeable to *in vitro* polarized Th17 cells, especially if the monolayer is pre-treated with IL-17 or IL-22 (Kebir et al., 2007). These data led to the hypothesis that in MS Th17 cells weaken the blood-brain barrier and enable the migration of immune cells into the normally immune privileged sites within the central nervous system. If this is the case, then Th17 cells may have more of a facilitative than directly pathogenic role in the nervous system, distinct from their clear role in the positive feedback loop of inflammation in psoriasis and RA. Recruitment has begun for a phase II clinical trials of Secukinumab in patients with relapsing-remitting MS. This trial will provide significant insight into the question of whether IL-17 blockade in MS can induce a clinically relevant protective function.

Type 1 diabetes (T1D) is characterized by autoimmune destruction of pancreatic islet cells resulting in the loss of insulin production. Murine studies have yielded conflicting results on the role of Th17 in the NOD mouse model of T1D (Vukkadapu et al., 2005; Jain et al., 2008; Emamaullee et al., 2009; van den Brandt et al., 2010; Lee et al., 2011; Liu et al., 2011; Joseph et al., 2012), including a potential ability to convert into Th1 cells *in vivo* (Bending et al., 2009; Martin-Orozco et al., 2009). However, more recent data suggest some of this apparent plasticity could be related to the study of *in vitro* polarized Th17 cells, which are not sufficiently stabilized at the epigenetic level (Bending et al., 2011; Cohen et al., 2011). Patients with T1D, have an increase in circulating IL-17-producing cells (Honkanen et al., 2010; Marwaha et al., 2010; Hughson et al., 2011), including FOXP3 expressing Th17 cells (Marwaha et al., 2010), monocytes that secrete Th17 polarizing cytokines (Bradshaw et al., 2009), and islet-antigen specific Th17 cells (Arif et al., 2011). There is also evidence that pancreatic lymph nodes from T1D patients have an increase in Th17 cells (Ferraro et al., 2011) and that islets from T1D patients, who died close to diagnosis, express IL-17A, RORC, and IL-22 (Arif et al., 2011). Mechanistically IL-17 enhances IL-1β, IFN-γ, and TNF-α-induced apoptosis in human islets (Arif et al., 2011). We have also found that significantly elevated levels of $CD8^{+}IL\text{-}17^{+}$ cells are detectable in the peripheral blood of a large subset of patients with T1D at disease onset (Marwaha et al., 2010). In the context of mounting correlative evidence that IL-17-producing cells may be pathogenic in the early stages of T1D onset, clinical trials to test the effects of therapy with agents such as Secukinumab or Ustekinumab are warranted.

A PROTECTIVE ROLE FOR Th17 IN THE GUT

The success of Ustekinumab, a human IL-12/23 monoclonal antibody, in patients with moderate to severe Crohn's disease held promise for the targeting of the IL-17 pathway to modulate this disease (Sandborn et al., 2008). However, the paradoxical exacerbation of Crohn's disease in the clinical trial of a Secukinumab, cast into doubt the pro-inflammatory role of Th17 cells in the gut. Whilst IL-17 cell-producing cells are found in high numbers in inflamed mucosa in Crohn's disease and Ulcerative colitis patients, more recent data demonstrate that characterization on the basis of IL-17 alone is insufficient to classify these cells as pathogenic. As described above, Th17 can co-secrete IFN-γ (Annunziato et al., 2007; Lee et al., 2009; Cosmi et al., 2011; Hirota et al., 2011) or co-express FOXP3 (Ayyoub et al., 2009; Beriou et al., 2009; Miyara et al., 2009; Voo et al., 2009; Kryczek et al., 2011; Ye et al., 2011), indicating the existence of multiple subsets of Th17 cells with functional specialization (**Figure 1**).

In retrospect, data from mouse models of colitis heeded a warning as to the protective role for Th17 cells in Crohn's disease. In the dextran sulfate sodium (DSS)-induced colitis model, administration of a neutralizing IL-17A antibody (Ogawa et al., 2004), deletion of IL-17A (Yang et al., 2008b), or of IL-22 (Zenewicz et al., 2008) all resulted in a worsening of the colitis. In contrast, IL-17F-deficient (Yang et al., 2008b) and IL-21-deficient mice (Fina et al., 2008) were protected against DSS induced colitis. These data suggest there are non-redundant roles of IL-17A versus F, and that, at least in the gut, IL-21 rather than IL-17A or IL-22 may be a primary Th17-derived pathogenic cytokine.

How could Th17-derived cytokines exert a protective functional role in the intestine? First, IL-17A improves barrier function

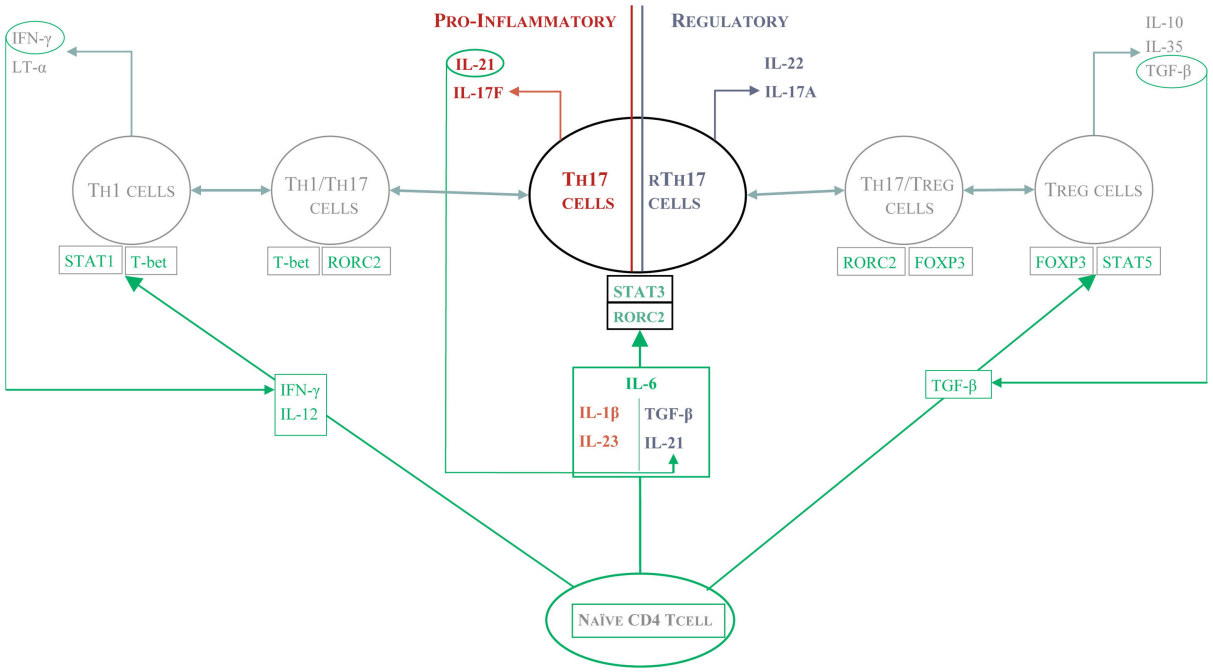

FIGURE 1 | The protective and pathogenic functions of Th17 cells. Depending on the local cytokine environment, different subsets of Th17 cells arise and mediate distinct effector function. In the presence of IL-23, Th17 cells seem to be pro-inflammatory and can either make IFN-γ themselves or work in concert with Th1 cells to drive a positive feedback pathway of tissue damage such as that seen in psoriasis or RA. In Crohn's disease, Th17 cells can differentiate in the gut into protective or pathogenic Th17 cells. In the presence of TGF-β and IL-21, Th17 are re-programmed into "regulatory" Th17 cells which seem to protect from intestinal inflammation.

by strengthening tight junctions after inducing claudin and mucin expression (Kinugasa et al., 2000; Chen et al., 2003). Second, IL-22 improves barrier function by inducing epithelial cell proliferation (Brand et al., 2006) and enhancing goblet cell restoration and mucus production (Sugimoto et al., 2008). Also, a novel suppressive Th17 subset dubbed regulatory Th17 (rTh17) cells has recently been described. When Esplugues et al. (2011) used a CD3-antibody strategy to induce mucosal tolerance, Th17 cells were recruited to the gut but then re-programmed into suppressive, FOXP3-negative, rTh17 cells. The function of rTh17 cells depends on IL-10, TGF-β, and CTLA-4, and does not occur in CCR6-deficient mice where Th17 are not recruited to the gut. The latter data indicate that the mucosal immunity micro-environment is critical for the development of rTh17 cells.

CONCLUSION

Different flavors of Th17 differentiation, ranging from highly pro-inflammatory to suppressive, result from different cytokine micro-environments in various diseases. Th17 cells can no longer be identified solely on the production of IL-17A since the combination of co-secreted cytokines is key to defining their effector function. Moreover, IL-17 is not only produced by Th17 cells, and under certain conditions γδ T cells (Stark et al., 2005), $CD8^+$ T cells (Shin et al., 1998; He et al., 2006), T follicular helper cells (Cua and Tato, 2010), Lymphoid Tissue induced (LTi) cells (Cupedo et al., 2009), and NKT cells (Michel et al., 2007; Lee et al., 2008; Rachitskaya et al., 2008), can all secrete IL-17. We must therefore start to redefine the partial role that Th17 cells play in IL-17-guided immune response. An additional consideration is their potential for plasticity and co-secretion of cytokines that define other Th cell lineages (e.g., IFN-γ), although this is more likely a transient rather than permanent change based on epigenetic analysis (Bending et al., 2011; Cohen et al., 2011). In summary, the notion that Th17 cells are purely pro-inflammatory cells is mistaken, rather these cells mediate a diverse set of responses in infection, autoimmunity, and immunodeficiency.

ACKNOWLEDGMENTS

The authors' own work is supported by grants from the Canadian Institutes for Health Research and the Juvenile Diabetes Research Foundation. Megan K. Levings holds a Canada Research Chair in Transplantation. Alicia N. McMurchy is a Canada Vanier Scholar. Ashish K. Marwaha holds a Michael Smith Research Trainee award and a Radcliffe Travelling Fellowship at the University of Oxford.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 April 2012; paper pending published: 21 April 2012; accepted: 04 May 2012; published online: 04 June 2012.

*Citation: Marwaha AK, Leung NJ, McMurchy AN and Levings MK (2012) TH17 cells in autoimmunity and immunodeficiency: protective or pathogenic? Front. Immun. **3**:129. doi: 10.3389/fimmu.2012.00129*

This article was submitted to Frontiers in Primary Immunodeficiencies, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 02 August 2012
doi: 10.3389/fimmu.2012.00233

Dendritic cells: a double-edge sword in autoimmune responses

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Dendritic cells (DC) are antigen-presenting cells that play a pivotal role in regulating innate and adaptive immune responses. In autoimmunity, DC act as a double-edged sword since on one hand they initiate adaptive self-reactive responses and on the other they play a pivotal role in promoting and maintaining tolerance. Thus, DC are the most important cells in either triggering self-specific responses or in negatively regulating auto-reactive responses. The latter function is mediated by DC in the steady-state or specialized subsets of DC, named tolerogenic DC. Clinical and experimental evidence indicate that prolonged presentation of self-antigens by DC is crucial for the development of destructive autoimmune diseases, and defects in tolerogenic DC functions contribute to eradication of self-tolerance. In recent years, DC have emerged as therapeutic targets for limiting their immunogenicity against self-antigens, while tolerogenic DC have been conceived as therapeutic tools to restore tolerance. The purpose of this review is to give a general overview of the current knowledge on the pathogenic role of DC in patients affected by autoimmune diseases. In addition, the protective role of tolerogenic DC will be addressed. The currently applied strategies to block immune activation or to exploit the tolerogenic potential of DC will be discussed.

Keywords: dendritic cells, autoimmune diseases, tolerance

INTRODUCTION

Dendritic cells (DC) are professional antigen-presenting cells (APC) specialized in capturing and processing antigens (Ags) to present to T cells. DC constitute a front-line defense against pathogens, are located throughout the body, and form complex networks that allow them to communicate with different cells. Therefore, DC are critically involved in the initiation of adaptive immune responses and, as such, are defined immunogenic DC. These DC might be implicated in the induction of autoimmune responses *via* the activation of auto-reactive T cells and the consequent eradication of self-tolerance. Conversely, DC in the steady-state, or specialized subsets of DC, termed tolerogenic DC, promote and maintain tolerance through several non-overlapping mechanisms. Tolerogenic DC can induce apoptosis of effector T cells, skew T cell phenotype, and promote anergy and/or regulatory T cells (Tregs; Morelli and Thomson, 2007; Gregori, 2011). Thus, defects in the activities of tolerogenic DC may also contribute to break self-tolerance and to induce autoimmune responses.

An optimal balance between immunogenic and tolerogenic DC is therefore fundamental to prevent self-reactive immune responses and to maintain immune self-specific homeostasis. In this review, we will give an overview of the different role of both immunogenic and tolerogenic DC in promoting autoimmune disease onset and/or progression, focusing primarily on human pathological conditions.

HUMAN DENDRITIC CELL SUBSETS

Dendritic cells are present in all tissues and they function as an important bridge between innate and adaptive immunity, by cellular interactions or through secretion of pro-inflammatory and immuno-regulatory cytokines (Banchereau and Steinman, 1998; Larregina and Falo, 2005; Merad et al., 2008; Rescigno and Di Sabatino, 2009; Lambrecht and Hammad, 2010; Thomson, 2010).

In the bloodstream, DC circulate as immature cells characterized by a low expression of human leukocyte antigen (HLA) class II and co-stimulatory molecules, high endocytic activity, and low T cell activation potential. Circulating DC constantly patrol the surrounding environment for pathogens, such as viruses and bacteria. Upon Ag encounter, DC undergo a complex process of maturation meanwhile they travel to the lymph nodes, where they activate helper and cytotoxic T cells as well as B cells. Immature DC in the steady-state migrate at low ratio to the lymph nodes without undergoing activation, can present Ags to T cells in the absence of co-stimulation and induce clonal T cell anergy (Schwartz et al., 1989), deletion of auto-reactive T cells (Hawiger et al., 2001; Steinman and Nussenzweig, 2002), and promote Tregs (Dhodapkar et al., 2001). Tolerogenic DC, both circulating and tissue resident, contribute to the induction and maintenance of self-specific tolerance.

In humans, two major and intrinsically different subpopulations of DC have been described: myeloid DC (myDC), called also conventional DC, and plasmacytoid DC (pDC), which differ in their transcriptional program, development, phenotypic markers, and immunological functions (Belz and Nutt, 2012). myDC pick up Ags in the periphery and move to T cell areas of peripheral lymphoid organs to initiate immunity through a number of different events including maturation and cytokine secretion, all of which

are regulated by recognition of pathogens *via* Toll-like receptors (TLR; Watts et al., 2010).

Myeloid DC are present in the peripheral blood and in several tissues where they acquire specialized functions. In the bloodstream, several subpopulations of immunogenic myDC, all of them expressing CD11c, and the myeloid markers CD13 and CD33, are present (**Table 1**). These cells include $CD16^+$ (they are also characterized by the expression of M-DC8; Schakel et al., 1999), BDCA-1^+, and BDCA-3^+ (Dzionek et al., 2001) that have different ability to stimulate allogeneic T cells (MacDonald et al., 2002). Distinct phenotypical and functional characteristics are displayed by myDC resident in peripheral tissues. These myDC can be distinguished according to the expression of specific markers: langerin (CD207) expressing cells (Geissmann et al., 2002; Larregina and Falo, 2005) are Langerhans cells (LC) and interstitial dermal DC localized in the skin; $CD103^+$ DC reside in the lamina propria (LP) of the small intestine (Jaensson et al., 2008; Rescigno and Di Sabatino, 2009); C-type $lectin^+$ (DC-SIGN) DC are present in the decidua (Laskarin et al., 2007); BDCA-1^+ and BDCA-3^+ DC have been described in the lung (Demedts et al., 2005; **Table 1**).

In addition to immunogenic myDC, other subsets of myDC with tolerogenic properties have been described such as DC expressing the scavenger receptor CD163 and immunoglobulin-like transcript 3 (ILT3; Maniecki et al., 2006). We recently identified DC-10, which are tolerogenic DC characterized by the expression of $CD11c^+$, $CD14^+$, $CD16^+$, $CD83^+$, and the tolerogenic molecules HLA-G and ILT4 (Gregori et al., 2010). DC-10 display a mature phenotype since they express both HLA class II and co-stimulatory molecules. They have a unique cytokine secretion profile consisting of high levels of IL-10 in the absence of IL-12 (Gregori et al., 2010). Specialized subsets of tolerogenic DC have been described in each tissue where they maintain tissue homeostasis and tolerance (reviewed in Gregori, 2011).

Plasmacytoid DC are component of the innate immune system and are specialized in producing interferon-α (IFN-α) upon activation *via* TLR7- and TLR9-mediated recognition of nucleic acids, and participate in T cell immunity (reviewed by Colonna et al., 2004). Similar to myDC, immature pDC as well as alternatively activated pDC are involved in promoting tolerance (Hanabuchi et al., 2010; Martin-Gayo et al., 2010). pDC are characterized by the expression of BDCA-2, BDCA-4 (Dzionek et al., 2001), IL-3R (CD123; Jahnsen et al., 2000), and ILT7 (Cao and Bover, 2010). pDC are found in the peripheral blood, lymph nodes, and the thymus, and they are recruited to sites of inflammation under pathological conditions (Swiecki and Colonna, 2010).

DENDRITIC CELLS IN CENTRAL AND PERIPHERAL TOLERANCE

To avoid autoimmune reactions, self-reactive lymphocytes have to be deleted or rendered tolerant. Several mechanisms are operating in the central and peripheral compartments to induce and maintain tolerance. Defects in these mechanisms are associated with the activation of immune responses against self-Ags (Goodnow et al., 2005). Central tolerance occurs in the thymus and leads to the deletion of self-reactive T cells through the positive and negative selection (Hogquist et al., 2005). The role of DC in central tolerance has become evident in the last decades. Thymic myDC are very efficient in mediating negative selection of developing thymocytes (Brocker et al., 1997; Ohnmacht et al., 2009). In addition, peripheral myDC can migrate to the thymus and contribute to negative selection (Bonasio et al., 2006; Proietto et al., 2008). Both thymic myDC and pDC play an important role in promoting positive selection of Tregs (Proietto et al., 2008; Hanabuchi et al., 2010; Martin-Gayo et al., 2010). Thus, myDC and pDC cooperate in the thymus to promote on one hand negative selection of self-reactive T cells, and on the other positive selection of Tregs.

To control immune responses to self-Ags that are not expressed in the thymus or may escape negative selection, different mechanisms of tolerance are operational in the periphery during the entire lifespan. Mechanisms of peripheral tolerance include cell death with consequent clonal deletion, development of a state of T cell unresponsiveness, and active suppression mediated by Tregs. DC, *via* the production of the immuno-modulatory cytokines IL-10 and TGF-β or the expression of the tolerogenic

Table 1 | Different subsets of human dendritic cells.

	Tissue distribution	Markers	Reference
Myeloid DC Immunogenic			
BDCA-1	Blood/tissues	CD11c, BDCA-1	MacDonald et al. (2002), Dzionek et al. (2000, 2001)
BDCA-3	Blood/tissues	CD11c, BDCA-3	MacDonald et al. (2002), Dzionek et al. (2000, 2001)
M-DC8	Blood/tissues	M-DC8, CD16	Schakel et al. (1999)
DC-SIGN	Blood/decidua	CD11c, DC-SIGN	Laskarin et al. (2007)
Langerhans cells	Skin	CD207, CD1a	Larregina and Falo (2005), Geissmann et al. (2002)
Myeloid DC tolerogenic			
CD163	Blood	CD11c, ILT3	Maniecki et al. (2006)
DC-10	Blood/tissues	CD11c, CD14, CD16, CD83, HLA-G, ILT4	Gregori et al. (2010)
CD103	Lamina propria	CD11c, CD103	Jaensson et al. (2008), Rescigno and Di Sabatino (2009)
Plasmacytoid DC			
pDC	Blood/tissues	BDCA-2, BDCA-4, CD123, ILT7	Dzionek et al. (2000, 2001), Jahnsen et al. (2000), Cao and Bover (2010)

molecules indoleamine 2,3-dioxygenase (IDO) or ILTs (Morelli and Thomson, 2007; Gregori, 2011), can regulate several of these processes.

ROLE OF DENDRITIC CELLS IN PRIMING AND SUSTAINING SELF-REACTIVE IMMUNE RESPONSES

In genetically susceptible individuals, autoimmune diseases may develop as a result of alterations in the expression of self-Ags by DC, or access to immune privileged sites, or modification of the activation state of DC that became potent activators/inducers of self-reactive effector T cells. Multiple evidences from pre-clinical models of autoimmune diseases indicate that DC loaded with self-Ags acquired an activated phenotype and are able to trigger autoimmune responses *via* the induction of T helper 1 (Th1) and Th17 responses (Torres-Aguilar et al., 2010). Priming of self-reactive T cells by activated DC that have taken up apoptotic cell debris may also lead to break-down self-tolerance and can result in autoimmunity (Lleo et al., 2008). The pro-inflammatory environment generally observed in organs target of autoimmunity can modify several tolerogenic DC functions, shifts the balance between tolerogenic and immunogenic DC toward the latter, and contributes to the development of autoimmune diseases.

Several factors in autoimmune patients indicate that the dysregulation in the immunogenic and tolerogenic DC is associated with excessive self-reactive responses and inflammation.

ABERRANT ACTIVATION OF IMMUNOGENIC DENDRITIC CELLS IN HUMAN AUTOIMMUNE DISEASES

In the last decades, studies of DC in patients indicate that aberrant DC activation or functions are associated with different autoimmune diseases as including Rheumatoid Arthritis (RA), Multiple Sclerosis (MS), Systemic Lupus Erythematosus (SLE), Psoriasis, and Inflammatory Bowel Disease (IBD; **Table 2**).

Rheumatoid Arthritis

In the peripheral blood of RA patients, but also in synovial fluids and tissues, increased numbers of myDC and pDC are present (Lebre and Tak, 2009). Studies on myDC from synovial fluid of RA patients show that these cells display an activated phenotype as they express high levels of HLA-DR and co-stimulatory molecules. Interestingly, myDC in inflamed tissues are associated with T cells in structures similar to germinal centers where they stimulate self-reactive T cells (Santiago-Schwarz, 2004). These myDC are also involved in promoting synovial inflammation due to their ability to secrete pro-inflammatory cytokines (Jongbloed et al., 2006; Lebre et al., 2008).

The role of pDC in the RA pathogenesis is dual: on one hand in synovial tissues pDC *via* the secretion of type I IFNs contribute to local inflammation, although at lower extend as compared to myDC (Pettit et al., 2000; Takakubo et al., 2008); on the other hand, pDC could play a role in activating B cells *via* the expression of B cell-activating factor (Lebre et al., 2008), leading to antibody production, which sustain tissue damage.

Multiple Sclerosis

The active participation of DC in the MS pathology is supported by their presence and activation in the central nervous system (CNS) of MS patients (Pashenkov et al., 2002). Increased frequency of myDC in the CNS at early stages of the disease and their presence within the demyelinating lesions indicate that myDC play a role in re-activating T cell responses to myelin upon entry into the CNS (Wu and Laufer, 2007). In addition to their identification in the CNS during the disease, analyses of myDC in the peripheral blood of MS patients revealed their ability to secrete pro-inflammatory cytokines at higher levels than DC from normal donors (Karni et al., 2006). These activated myDC, polarize $CD4^+$ T cells toward IFN-γ-producing effector cells (Karni et al., 2006; Vaknin-Dembinsky et al., 2006). Thus, myDC in MS patients are highly immunogenic and contribute to disease induction and progression.

The role of pDC in the MS pathogenesis is less clear. No differences in the absolute number of pDC have been found in the peripheral blood of MS patients. However, a reduced stimulatory activity of pDC and a limited expression of co-stimulatory molecules upon *in vitro* activation were described, suggesting an impairment in the maturation and an altered regulatory functions of pDC in MS patients (Stasiolek et al., 2006).

Systemic Lupus Erythematosus

The induction of SLE and disease severity is associated with a defect in clearance of apoptotic cells by macrophages (Herrmann et al., 1998). This results in hyper-activation of DC and leads to the chronic inflammation observed in SLE (Seitz and Matsushima, 2010). When apoptotic cells are not rapidly removed, they release blebs, in which SLE auto-Ags are clustered, and induce maturation of DC. These DC can stimulate the production of IL-2, IFN-γ, and, in particular, IL-17 by T cells that sustain autoimmune responses (Fransen et al., 2009). Although myDC are reduced in the peripheral blood of SLE patients (Robak et al., 2004; Migita et al., 2005), they contribute to effector T cell activation because of their activated phenotype (Ding et al., 2006; Gerl et al., 2010). In line with this notion, monocytes from SLE patients undergo an accelerated differentiation *in vitro* and express high levels of co-stimulatory molecules (Ding et al., 2006; Gerl et al., 2010).

Plasmacytoid DC are also reduced in the peripheral blood of SLE patients (Robak et al., 2004; Migita et al., 2005), but they accumulate in inflamed skin lesions (Mori et al., 1994) where they are selectively attracted by ChemR23, the chemokine receptor for chemerin (Vermi et al., 2005). Moreover, circulating pDC from SLE patients migrate in response to CCL19 (Gerl et al., 2010). It has been proposed that the increased responsiveness to CCL19 might lead to pDC accumulation in T cell area of lymph nodes where they increase the priming of self-reactive T cells and contribute to SLE pathogenesis (Gerl et al., 2010).

Psoriasis

In psoriatic lesions, the frequency of myDC is 30-fold increased with respect to normal skin (Zaba et al., 2007). The large proportion of these cells secretes TNF-α, IL-12, IL-23, and the inducible nitric oxide synthase (iNOS; Lowes et al., 2005). These cytokines activate keratinocytes and fibroblasts to secrete pro-inflammatory cytokines (IL-6 and IL-1) that induce effector Th1 and Th17 cells, contributing to dermal inflammation and epidermal hyperplasia characteristic of psoriasis (Zheng et al., 2007; Pene et al., 2008).

Table 2 | Role of dendritic cell subsets in human autoimmune diseases.

Disease	DC subsets	Localization	Function	Reference
Rheumatoid Arthritis	myDC	Blood/synovial tissues	↑ Effector T cell priming ↑ Pro-inflammatory cytokines ↑ DC activation	Santiago-Schwarz (2004), Takakubo et al. (2008)
	pDC	Synovial tissues	↑ IFN-α production ↑ B cell activation	Takakubo et al. (2008), Lebre et al. (2008)
Multiple Sclerosis	myDC	Cerebrospinal fluid	↑ T cell activation ↑ Pro-inflammatory cytokines	Pashenkov et al. (2002), Wu and Laufer (2007), Karni et al. (2006)
	pDC	Blood	Impairment in maturation ↓ Ability to prime FOXP3$^+$ Tregs	Stasiolek et al. (2006)
Systemic Lupus Erythematosus	myDC	Blood	↑ DC activation ↑ Effector Th1/Th17 cell priming	Seitz and Matsushima (2010), Fransen et al. (2009)
	pDC	Inflamed lesions/LN	↑ Effector T cell priming	Robak et al. (2004), Migita et al. (2005), Gerl et al. (2010)
Psoriasis	myDC	Psoriatic lesions	↑ Pro-inflammatory cytokine ↑ Effector T cell priming	Lowes et al. (2005), Zheng et al. (2007)
	pDC	Psoriatic lesions	↑ IFN-α production	Nestle et al. (2005)
Inflammatory Bowel Disease	myDC	Inflamed lesions	↑ DC activation ↑ Pro-inflammatory cytokines ↑ T cell activation	te Velde et al. (2003), Hart et al. (2005)
	pDC	Lamina propria	↑ DC activation ↑ IFN-α production ↓ TNF-α production	Baumgart et al. (2011)

The frequency of IFN-α-secreting pDC is also increased in psoriatic lesions and participate to local inflammation (Nestle et al., 2005).

Inflammatory Bowel Disease

Several studies in Crohn's disease (CD) and ulcerative colitis (UC) patients have demonstrated an abnormal intestinal accumulation of DC expressing BDCA-1, which contribute to excessive T cell activation (de Baey et al., 2003; te Velde et al., 2003; Silva et al., 2004). DC from CD patients have an altered cytokine production profile since they produce higher levels of IL-12 and IL-6 than DC from healthy donors (Hart et al., 2005). Thus, myDC accumulate in the intestine of IBD patients where they activate pathogenic T cells.

It has been recently reported that pDC might participate to inflammation in the mucosa of CD and UC patients. Indeed high frequency of pDC was found in inflamed mucosa of CD and UC patients. Studies on pDC from the peripheral blood of flaring CD and UC patients demonstrated that they express higher levels of CD40 and CD86, and they secrete higher amounts of TNF-α than pDC from healthy subjects. However, these pDC were impaired in their ability to secrete IFN-α (Baumgart et al., 2011). Thus, these results suggest that aberrant activation of pDC or alteration in their regulatory functions could play a role in the pathogenesis of IBD.

These examples clearly indicate that hyper-activation of myDC is one of the key factors in promoting self-reactive T cell immunity. Moreover, an aberrant pDC distribution and function contribute to the local inflammation in target organs of autoimmunity. In this scenario, activated DC are recruited to the inflamed tissues where they secrete pro-inflammatory cytokines (i.e., IL-1, TNF-α, IFN-α, and IL-6) or express high levels of co-stimulatory molecules that induce an immune-stimulatory loop causing re-activation of self-reactive T cells and recruitment and/or the activation of other immune cells, including additional DC.

ALTERATION OF TOLEROGENIC DENDRITIC CELL FUNCTIONS AND AUTOIMMUNITY

In homeostatic and resting conditions (in the absence of inflammation) DC preserve an immature or semi-mature phenotype,

and actively participate in the maintenance of tolerance toward self-Ags. In these conditions, tissue resident tolerogenic DC control self-reactive T cell responses by preventing excessive local inflammation and autoimmune-mediated tissue damages. The presence of high levels of pro-inflammatory mediators observed in chronic inflamed tissues decreases the regulatory activity of tolerogenic DC.

One of the most important features of tolerogenic DC is their ability to secrete immuno-regulatory cytokines, such as IL-10 and TGF-β. IL-10 directly suppresses T cell responses by inhibiting the secretion of IL-2 and IFN-γ (Vieira et al., 1991) and by preventing T cell proliferation (Taga and Tosato, 1992). Similarly, TGF-β potently inhibits T cell responses (Gorelik and Flavell, 2002). IL-10 controls a number of different cells implicated in inflammatory responses, including APC (Mosser and Zhang, 2008). The expression of HLA class II, co-stimulatory molecules (de Waal Malefyt et al., 1991) and pro-inflammatory cytokines (Fiorentino et al., 1991a,b) is down-regulated by IL-10. On the other hand, IL-10 up-regulates the expression of tolerogenic molecules such as ILT3 and ILT4 (as reviewed in Suciu-Foca et al., 2005), and HLA-G (Moreau et al., 1999) on APC, rendering them capable of dampening immune responses and inducing Tregs (Carosella et al., 2011). In the steady-state, DC secrete high levels of IL-10, can modulate the activation of neighboring myDC, and promote the *de novo* induction of tolerogenic DC. *In vitro* studies demonstrated that maturation of monocytes derived DC in the presence of exogenous IL-10 is inhibited, and resulting DC become able to induce anergic/suppressive T cells (Steinbrink et al., 1997, 2002). Moreover, differentiation of monocytes derived DC in the presence of IL-10 results in a population of mature myDC, called DC-10, which secrete high levels of IL-10 and are potent inducers of Ag-specific IL-10-producing type 1 regulatory (Tr1) cells *in vitro* (Gregori et al., 2010; Pacciani et al., 2010). In addition to their ability to secrete high levels of IL-10, DC-10 strongly express ILT4 and HLA-G, which are necessary for efficient Tr1 cell induction. In inflamed tissues, high amounts of pro-inflammatory cytokines lead to the down-regulation of IL-10 production that could impair the modulation of already differentiated DC, and the *de novo* induction of tolerogenic DC, including DC-10.

It has been reported that mutations in IL-10 or in its receptor lead to the loss of IL-10 function and cause severe intractable infant and adult enterocolitis (Glocker et al., 2009, 2010), demonstrating the critical role of IL-10 in maintaining intestinal tolerance. More recently, it has been shown that DC generated from peripheral monocytes of IBD children carrying a mutation in IL-10R secrete significantly higher amounts of TNF-α, IL-12, and IL-23 than DC from healthy controls (Begue et al., 2011). These data indicate that impairment in the ability of DC to produce IL-10 and to respond to it is critically involved in the pathogenesis of IBD.

In addition to soluble factors, tolerogenic DC can express immuno-regulatory enzymes such as IDO and heme oxygenase-1 (HO-1), which suppress T cell responses and promote immune tolerance. IDO inhibits effector T cell proliferation by reducing tryptophan that is necessary for cell division (Mellor and Munn, 2004). HO-1 is the rate-limiting enzyme in heme catabolism and it acts as an anti-inflammatory molecules, controlling apoptosis, T cell proliferation and activation (Otterbein et al., 2000; Pae et al., 2004). In non-pathological conditions, $Foxp3^+$ Tregs promote IDO expression in myDC through the interaction of cytotoxic T-lymphocyte antigen 4 (CTLA-4) with CD80 and CD86 (Fallarino et al., 2002, 2003; Grohmann et al., 2002). Resulting myDC acquire the ability to generate $Foxp3^+$ Tregs (Mellor and Munn, 2004). During inflammation, chronically activated myDC, although expressing high levels of CD80 and CD86, become refractory to the inhibitory signal induced by $Foxp3^+$ Tregs and unable to express IDO.

Indoleamine 2,3-dioxygenase can also be expressed by pDC alternatively activated with anti-CD40L and IL-3 (Martin-Gayo et al., 2010) or with thymic stromal lymphopoietin (TSLP; Hanabuchi et al., 2010). These IDO expressing pDC have been shown to promote the induction of $Foxp3^+$ Tregs. In the synovial fluid of RA patients, IDO expressing pDC have been identified (Takakubo et al., 2008), but their limited number and the presence of an increased frequency of activated myDC impair their ability to counteract self-reactive effector T cell responses by the induction of Tregs.

Immune cells and non-immune cells can play an important role in driving the development of tolerogenic DC. It has been shown that human intestinal epithelial cells (IECs) through the secretion of TSLP, TGF-β, and retinoic acid drive the development of $CD103^+$ tolerogenic DC (Iliev et al., 2009). $CD103^+$ DC promote the *de novo* induction of $Foxp3^+$ Tregs and inhibit Th1 and Th17 responses (Iliev et al., 2009). In CD patients, IECs do not express TSLP and fail to control DC-mediated pro-inflammatory responses, resulting in abnormal release of IL-12 (Rimoldi et al., 2005) and reduced ability to induce $CD103^+$ DC (Rescigno and Di Sabatino, 2009). This perturbation in the cross-talk between IECs and DC disrupts the intestinal immune-homeostasis and promotes gut inflammation.

In conclusion, chronic inflammation and the presence of high levels of pro-inflammatory cytokines in target organs of autoimmunity and in the periphery alters the regulatory activity of tolerogenic DC and generate an imbalance between tolerogenic and immunogenic DC, which sustains constant activation of self-reactive T cells leading to tissue damage.

STRATEGIES TO PROMOTE TOLERANCE BY TARGETING DENDRITIC CELLS

Autoimmune diseases are the result of a potent and de-regulated immuno-responses toward self-Ags mediated by a variety of immune cells, including B and T lymphocytes, and APC. The critical role of DC in the initiation and in the progression of autoimmune diseases indicates that DC targeting therapies could represent a good alternative to current immuno-modulatory therapies already approved for the treatment of autoimmune diseases. Two alternatives approaches can be foreseen to modulate DC: (i) therapies targeting immunogenic DC to lower their activation, (ii) therapies targeting tolerogenic DC to improve their function and induction.

Treatment with monoclonal antibodies (mAb) against pro-inflammatory cytokines or their receptors aiming to reduce the DC immunogenicity are currently under clinical investigation for the

treatment of autoimmune diseases. Administration of Anakinra, a recombinant version of IL-1Rα, in combination with methotrexate (MTX), or of Tocilizumab, a humanized mAb that competes with IL-6 for receptor binding, provided good clinical benefit in RA patients (Smolen et al., 2008; Niu et al., 2011). Positive results were obtained also in patients with RA, CD, and psoriasis treated with anti-TNF-α mAb (Infliximab; Present et al., 1999; Cohen et al., 2000; Ricart et al., 2001). Two recent phase II clinical trials, proved the efficacy and safety of a two different mAbs against IL-17 (Ixekizumab; Leonardi et al., 2012) or its receptor (Brodalumab; Papp et al., 2012) for the treatment of Psoriasis. Despite these encouraging results, additional studies are needed to evaluate the safety of long-term treatment with these mAbs and to define the optimal schedule for their efficacy. Notably, to obtain stable clinical benefit, chronic administration of these mAbs is required since clinical symptoms return after treatment withdrawal.

An alternative approach to block DC immuno-stimulatory activity is the inhibition of co-stimulatory molecules (CD80 and CD86). In pre-clinical models of autoimmune diseases the efficacy of CD28/B7 blockade by CTLA-4Ig has been shown (Salomon and Bluestone, 2001). Interestingly, while the efficacy and tolerability of CTLA-4Ig (Abatacept) have been reported across multiple international, randomized, double blind, placebo control trials in patients with active RA (Massarotti, 2008), its effect in other autoimmune diseases, such as Psoriasis and MS, is still not clear (Sakthivel, 2009) and additional investigations are required.

Results from these clinical trials indicate that therapies with mAb aim at inhibiting pro-inflammatory cytokines or co-stimulatory signaling pathways are efficacious; however, they required long-term administration with consequent long-term detrimental effects for patients.

Another alternative strategy to restore tolerance in autoimmunity is to improve the induction and function of tolerogenic DC. The majority of the efforts have been focused on generating tolerogenic DC *in vitro* to be subsequently administered *in vivo* as cell therapy, rather than in promoting *in vivo* the expansion of tolerogenic DC. Different immune-modulatory agents have been used in order to modify the phenotype, cytokine profiles and activity of DC. Encouraging results have been obtained by treating DC with biological agents such as dexamethasone (Piemonti et al., 1999) or vitamin D3 (Penna and Adorini, 2000) or cytokines such as TNF-α (van Duivenvoorde et al., 2004, 2007) or IL-10 (Steinbrink et al., 1997, 2002; Sato et al., 2003; Gregori et al., 2010). In pre-clinical models of arthritis (van Duivenvoorde et al., 2004, 2007), EAE (Menges et al., 2002), and type 1 diabetes (T1D; Feili-Hariri et al., 2002) the efficacy of *in vitro* induced tolerogenic DC-based cell therapy has been demonstrated. In addition, repetitive injection of immature DC has been shown to protect mice from collagen-induced arthritis (Charbonnier et al., 2006). To date, in the field of autoimmune diseases, no data have been published using immuno-modulatory pDC as therapeutic tools.

Despite the fact that *in vitro* generated human tolerogenic DC have been studied in research settings, the described methods have not been translated into clinical grade protocols. Recently a comparative analysis of good manufacturing practice protocols to generate human tolerogenic DC using IL-10, TGF-β, vitamin D3, dexamethasone or rapamycin has been performed (Boks et al., 2012). Results from this study demonstrated that DC activated in the presence of IL-10 (IL-10 DC) showed the most powerful tolerogenic characteristics with high IL-10 production and low T cell activation. Based on these results the authors suggested that IL-10 DC are the best suitable subset of tolerogenic DC for tolerance inducing therapies. We developed a protocol to generate human tolerogenic DC by differentiating monocyte derived DC in the presence of exogenous IL-10. Resulting cells, called DC-10, represent a powerful subset of tolerogenic DC. DC-10 are phenotypically and functionally stable and upon activation they maintain their cytokine production profile (high IL-10/IL-12 ratio) and their ability to differentiate adaptive Ag-specific Tr1 cells (S. Gregori, personal communication). In alternative to IL-10, a method to generate clinical grade tolerogenic DC from patients with RA using vitamin D3 and dexamethasone has been also developed (Harry et al., 2010) and a clinical trial for treating RA patients will be initiated soon (Moreau et al., 2009). Results from this first proof of principle clinical trial will provide informations on the safety and efficacy of tolerogenic DC-based cell therapy to restore tolerance in autoimmune settings.

CONCLUSIONS AND PERSPECTIVES

Over the past years significant progresses have been achieved in understanding the pathological role of DC in autoimmune diseases and how tolerogenic DC regulate and maintain tolerance toward self-Ags. Although a number of questions still remain to be addressed, inhibition of the immunogenic branch of DC function or induction of the tolerogenic one has become a feasible approach to restore tolerance in autoimmune diseases. Current approaches based on the administration of mAb against immunogenic proteins have been successful, however the lack of information regarding long-term safety and the chronic infusion limited their broaden application. Alternatively, *in vitro* differentiated tolerogenic DC are of great potential interest as cell therapy for there-establishment of immunological tolerance in autoimmune diseases. Nevertheless, the optimal type of tolerogenic DC still remains to be defined. It has to be taken into account that tolerogenic DC should be resistant to maturation either induced by *in vivo* transfer or by inflammatory mediators. Moreover, the route and dose of administration as well as the need of *in vivo* pharmacological treatments for maintaining their tolerogenic functions have to be still determined. Further studies in humanized mouse model as well as in large animals will elucidate these aspects and will allow the establishment of protocols with tolerogenic DC-based cell therapy for clinical application in autoimmune diseases.

ACKNOWLEDGMENTS

This work was supported by Telethon Foundation "Comitato Telethon Fondazione Onlus" Core Grant OSR-TIGET project E2 (Rome) and by the Italian Ministry of Health. Dr. Giada Amodio conducted this study as partial fulfillment of her PhD in Molecular Medicine, Program in Basic and Applied Immunology, San Raffaele University, Milan, Italy.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 April 2012; paper pending published: 27 May 2012; accepted: 15 July 2012; published online: 02 August 2012.

Citation: Amodio G and Gregori S (2012) Dendritic cells: a double-edge sword in autoimmune responses. Front. Immun. ***3****:233. doi: 10.3389/fimmu.2012.00233*

This article was submitted to Frontiers in Primary Immunodeficiencies, a specialty of Frontiers in Immunology.